HEALTH ASSESSMENT IN THE BOWHEAD WHALE

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

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May 2006

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Abstract

Tissue samples and morphometric data were collected from 64 bowhead whales landed during the 1998-2002 subsistence hunts in Barrow and Kaktovik, Alaska. Our primary goal was to assess the health status of the Bering-Chukchi-Beaufort Seas stock of bowhead whales. Ages of whales were determined via aspartic acid racemization of the eye lens nucleus, baleen stable carbon isotope analysis and morphometric and histologic indices. We investigated the gross and microscopic anatomy of organs and blubber, thyroid hormone concentrations, serum haptoglobin, vitamin A and E concentrations in liver, blubber and serum and essential element concentrations in liver and kidney. Thyroid hormone and vitamin A were also evaluated as potential biomarkers of organochlorine (OC) concentrations in blubber, liver and serum. Neither of these substances was found to correlate with the relatively low OC concentrations found in these mysticetes. Histological changes of interest included renal interstitial fibrosis, hepatic periportal fibrosis/pigmentation/lipidosis, splenic extramedullary hematopoiesis and pulmonary fibromuscular hyperplasia. Changes in the kidney and lung were related to both age and renal and hepatic Cd concentrations. Most of the histological differences observed did not appear to adversely affect organ function or health of the individual. Thyroid hormone concentrations were stable over age/sex/seasonal groups, however, pregnant females had significantly lower total and free thyroxine than non-pregnant adult females and other age-sex classes. Serum haptoglobin was measured as an indirect determinant of acute inflammation, with three reactors found among 51 whales examined. Liver contained the highest mean concentrations of vitamins A and E (followed by epidermis, blubber, and serum and serum, epidermis, and blubber, in order). Finally, blubber percent collagen was measured at 30 locations on each whale and was found to be stable by site and most depths, with the most internal region of the reticular dermis being the only exception.

Overall, the bowhead whales were healthy. However, climate change, offshore development and increases in arctic pollution emphasize the importance of baseline data collection. An ongoing surveillance effort is recommended to ensure that the species will be viable for generations to come and to assure subsistence users of the robust and healthy status of this stock of whales.

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List of Abbreviations and Acronyms

AAR	aspartic acid racemization
AEWC	Alaska Eskimo Whaling Commission
Ag	silver
AGEs	advanced glycation end-products
AMAP	Arctic Monitoring and Assessment Programme
ANOVA	analysis of variance
ANCOVA	analysis of covariance
BCBS	Bering-Chukchi-Beaufort Sea
BCP	blubber collagen percentage
BD	blowhole dorsal
BHA	butylated hydroxyanisole
BL	blowhole lateral
BPMO	benzo(a)pyrene monooxygenase
BV	blowhole ventral
CCA	cannonical correspondence analysis
Cd	cadmium
CIFAR	Cooperative Institute for Arctic Research
CML	carboxy-methyl lysine
Cu	copper
CV	coefficients of variation
dd	doubly distilled
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EFI	epithelial-follicular index
EH	epithelial height
EMH	extramedullary hematopoiesis

Fe	iron
FL	follicular length
fT3	free triiodothyronine
fT4	free thyroxine
FW	follicular width
GLM	general linear model
H&E	hematoxylin and eosin
HPLC	high-performance liquid chromatography
ICP-MS	inductively coupled plasma mass spectrometry
INBRE	Alaska IDeA Networks for Biomedical Research Excellence
IS	internal standard
IWC	International Whaling Commission
k_{Asp}	racemization rate
KOH	potassium hydroxide
MMHSRP	Marine Mammal Health and Stranding Program
MTH	metallothionein
NOAA	National Oceanic and Atmospheric Administration
NSB-DWM	North Slope Borough Department of Wildlife Management
OC	organochlorine
PBS	phosphate buffered saline
PCA	principle component analysis
PCBs	poly-chlorinated biphenyls
POPs	persistent organic pollutants
PUFA	poly-unsaturated fatty acid
RIA	radioimmunoassay
SD	standard deviation
Se	selenium

SE	standard error
T3	triiodothyronine
T4	thyroxine
TH	thyroid hormone
tHg	total mercury
tT3	total triiodothyronine
tT4	total thyroxine
TTR	transthyretin
UAF	University of Alaska Fairbanks
UD	umbilicus dorsal
UL	umbilicus lateral
UV	umbilicus ventral
Zn	zinc

Acknowledgements

This thesis is the result of six years of work whereby I have been accompanied and supported by many people. I am pleased to now have the opportunity to express my gratitude to all of them.

The first person I would like to thank is my advisor John Blake. I have been his student since 2000 when I started the combined veterinary residency/PhD program. During these years I have known John to be a patient and diplomatic person and an excellent veterinarian. His enthusiasm and integral view on research and wildlife medicine have made a deep impression on me. I owe him much gratitude for providing me with many amazing opportunities. I also thank Dr. Todd O'Hara who introduced me to northern Alaska and kept an eye on the progress of my work, and the other members of my PhD committee, Mike Castellini and Doug DeMaster, who monitored my work and took effort in reading it and providing me with valuable comments on earlier versions of this thesis. Matt Gray, Ron Barry and Dana Thomas were also of great assistance with statistics. I thank you all.

The staff at Animal Quarters gave me the feeling of being at home at work. To Chris Terzi, Don Hartbauer, Rob Aikman, Ron Standlee-Strom and Rhonda Swor (among others): many thanks for your patient support and willingness to help with whatever I asked for without complaint. I would also like to recognize the Department of Biology and Wildlife and the Graduate School, which were both of great assistance during my time at UAF.

I had the pleasure to work with several students who did their graduate work at the Institute of Arctic Biology and have been beneficial to the work presented in this thesis. I owe a magnitude of thanks to Lara Dehn, for her friendship, assistance (many samples processed), listening skills and support. Gay Sheffield was also helpful in providing samples from Kaktovik, friendship, humor and delicious beverages when needed (not necessarily in that order). You are both superwomen! Long live the B.O.E.!

This study would not have been possible without the help of the subsistence hunters in Barrow and Kaktovik, Alaska. I thank them from the bottom of my heart and hope that the results of my research are helpful to them. This research has been supported and funded by various organizations including the North

Slope Borough Department of Wildlife Management, Cooperative Institute for Arctic Research (CIFAR), Experimental Program to Stimulate Competitive Research (EPSCoR) and IDeA Networks for Biomedical Research Excellence (INBRE). I thank them all for their confidence in me. I am also grateful for aid provided by the UAF Stable Isotope Laboratory- specifically, Norma Haubenstock, Tim Howe and Tammy Massie for performing excellent work during the past years and cheerful assistance. I am grateful to Victoria Woshner, Craig George, Cyd Hanns, Benny Akootchook, Charlie Brower, Harry Brower and Taqulik Hepa who helped introduce me to Barrow, its science, its people and its culture. It was a pleasure to work with them and the other employees of the North Slope Borough Department of Wildlife Management and I look forward to working with them in the future.

In my opinion, doing a PhD is a sacred task and this was definitely one of the best decisions of my life. Special thanks go to Melissa Beckman, Debra Miller, Fawn Bassett and Marty Reedy, whom I have known for more than ten years now and who have been kind, helpful and trustworthy friends.

I feel a deep sense of gratitude towards my father and mother who have supported me through many years of school and have always encouraged me to attain my goals.

I am very grateful for my husband Ken, for his love and patience during the PhD period. I could not have done this without him.

My thanks would be incomplete without acknowledging God, Alaska and the bowhead whale. I am eternally grateful for the opportunities each have provided me. In the words of Henry Miller: Every man has his own destiny: the only imperative is to follow it, to accept it, no matter where it leads him.

CherylRosa

March 19, 2006
Fairbanks, Alaska

Chapter One

Introduction

Wildlife health assessment is a systematic approach involving the application of diagnostics and biological investigation to determine the health of a population by examining many individuals, determining baseline health parameters then making broader inferences about the health of the population. With this information one can infer ecological threats to the species and thus threats to humans, such as subsistence users, who depend upon wildlife resources. Health assessment can also be an effective means of emerging disease surveillance. Stressors in the environment, such as contaminants and human disturbance, can affect both individuals and populations. Examples include the endangered Northern right whale (*Eubalaena glacialis*), a species that is being heavily impacted by ship strikes and rope entanglement (Moore et al., 2004), and harbor porpoises (*Phocoena phocoena*) from the German North and Baltic Seas which may be experiencing contaminant-induced immunosupression from high exposure to PCBs (Beineke et al., 2005). Wildlife health assessment is a complex process requiring a comparative approach involving many disciplines, including, but not limited to, population biology, toxicology, physiology and ecology. However, although a comparative interdisciplinary approach can be effective, it must be used with caution in marine mammals because they have many specific adaptations unique to their marine existence.

The bowhead whale (*Balaena mysticetus*) is an endangered mysticete and an important subsistence species to many native communities in Alaska, Canada and Russia. The range of the bowhead whale includes arctic and subarctic waters. The Bering Chukchi Beaufort Seas (BCBS) stock has a yearly migration covering a route that stretches from the Bering to the Beaufort Sea, making it a potential indicator of the health of the Western Arctic marine ecosystem. This species is known for its exceptional longevity (possibly greater than 150 years) (George *et al.*, 1999) and feeds on a low trophic level (primarily zooplankton) (Lowry, 1993). It has an intimate association with the ice-edge and other highly productive areas in the Arctic. The BCBS stock of bowhead whales is currently growing, with the population presently estimated to be between 10,000 and 12,000 whales, with an estimated yearly population increase of ~3% since 2001 (George et al., 2004). The BCBS stock of bowhead whales is most likely between 30-80% of its carrying capacity (K), which has an effect on health, as animals in populations significantly below K have a

tendency to be in better health than those under greater resource stress or closer to K (Eberhardt *et al.*, 1977, Gerrodette and DeMaster, 1990, George *et al.*, 2004). Conducting health assessments in free-ranging cetaceans poses many logistical challenges. The bowhead whale subsistence hunt and the cooperation of the whaling captains provided the unique opportunity to collect samples from freshly killed whales over a number of years and from different age, gender and seasonal groups.

Marine mammals are potential sentinels of marine ecological disturbance in many cases, including pollutants in the marine ecosystem (Ross, 2000). The effects of global climate change are occurring in the Arctic (Hinzman *et al.*, 2005). Evaluation of the potential effects of global climate change on the sea ice and productivity of the Arctic and, in turn, on the marine and terrestrial species that live there is critical to health assessment efforts. Anticipated variations in temperature, sea ice thickness and prey abundance/distribution may affect the migration, range and health of bowhead whales. Industrial development, including offshore oil exploration (often seismic), noise and environmental contamination that may follow this activity, is increasing in the Arctic. Cetacean response to noise and disturbance is documented (Richardson and Malme, 1993), and long-term evaluation of the exposure and effects of contaminants is critical to health assessment efforts in areas undergoing industrial development. Additionally, the bowhead whale is an important subsistence food both nutritionally and culturally. This emphasizes the importance of baseline knowledge of this species, especially in a rapidly changing environment.

Natural and anthropogenic contaminants are of specific concern in the Arctic subsistence-based communities. The toxic effects of metals and persistent organic pollutants (POPs) and their environmental and food chain transport have been the subjects of recent studies in arctic regions (Mackey *et al.,* 1996; Wagemann *et al.,* 1998; Skaare *et al.,* 1996; Woshner *et al.,* 2001; Dehn *et al.,* 2005a, b). Anthropogenic contaminants impact the fauna of the Arctic and potentially the consumers of these animals (DeSwart et al., 1996, Hall et al., 1997, Beckmen et al., 2003). Most metals can affect multiple organ systems, but frequently each has a critical effect seen in a specific organ or tissue. Their effects are dose and route dependent.

Different marine mammal species bioaccumulate metals in different manners and exhibit different effects from toxic exposures. In the Arctic, the primary odontocete species studied include the beluga whale (*Delphinapterus leucas*) and the narwhal (*Monodon monoceros*). These whales occupy the top of the food chain, being mainly piscivorous. Bowhead whales are mysticetes with a range primarily in the Arctic. The species preys upon a low trophic level, feeding on zooplankton. Bratton *et al.* (1997) and O'Shea and Brownell (1994) showed that, in general, mysticete whales have lower concentrations of metal residues in their tissues than odontocetes, with the exception of cadmium (Cd).

In comparison to odontocete species, bowhead whales tend to have higher renal Cd concentrations, where accumulation increases relative to the age of the animal (Bratton *et al.*, 1997; Woshner *et al.*, 2001). To date, there has been no cadmium-induced pathology linked to these observed levels. In the bowhead whale, the mean concentration of Cd was highest in kidney (Bratton *et al.*, 1997). Mean hepatic Cd concentration was significantly lower than in kidney, while Cd level in muscle, blubber and epidermis were significantly lower than liver and kidney (Woshner et al., 2001).

The establishment of baseline histological parameters and the development of histological indices to couple changes at the ultrastructural level with elemental concentrations and other potential stressors is key to understanding their effects on the whale. Biomarkers have been evaluated in marine mammals, including cytochrome P450 isozyme induction (Angell *et al.*, 2004; Hahn *et al.*, 2000), c-reactive protein (Funke *et al.*, 1997), vitamin A (Simms and Ross, 2000), benzo(a)pyrene monooxygenase (BPMO) activity (Fossi *et al.*, 2003) and serum thyroid hormones (Schumacher *et al.*, 1993; Hall *et al.*, 1998, 2003). These all have potential value in the estimation of health in cetaceans.

Nutritional indices, including blubber (quality and quantity) and tissue vitamin and mineral levels, are also important to health assessment. Seasonal changes in body condition must be interpreted and understood in order to discriminate potentially normal fluctuations from nutritional stress, which may result in changes on both gross and biochemical levels. Changes to blubber may include a reduction in fat, protein and, in extreme cases, thickness (Koopman *et al.*, 2002). This is often related to resource availability and health in general (Koopman *et al.*, 2002; Moore *et al.*, 2004).

The confounding effect of age is critical to the interpretation of many, if not all, of the facets of marine mammal health assessment. Data such as element, hormone, and vitamin concentrations must be interpreted within the context of age. Age is also important in the histological interpretation of tissues. Cetacean age may be determined by various methods, ranging from photo identification to ear plug and tooth growth layer quantification, aspartic acid racemization in the teeth and eye lens nucleus and, to a limited extent in many cases, the aging of baleen. In bowhead whales, several aging techniques have been investigated. Of these, only two have been found to be dependable: aspartic acid racemic analyses of the eye lens nucleus and baleen stable isotopic aging.

This research will provide techniques and baseline data that can be applied to other threatened and endangered marine mammal populations, such as North Pacific humpback (*Megaptera novaeangliae*) and fin whales (*Balaenoptera physalus*) and the Northern right whale. It also provides baseline data on contaminant levels in an Alaskan native food source, along with a "normal" histologic assessment.

Objectives of this study:

1. To determine the normal range of values (natural variability due to time of year landed, age, and sex) for basic biological, nutritional and health parameters (blubber characteristics, essential and non-essential elements, as well as the microscopic anatomy of selected tissues) in the bowhead whale.

2. To describe the unique histological and histopathological findings from bowhead whale tissues and investigate the relationship between these histopathological findings and age, season, sex, and heavy metal and mineral status.

3. To analyze serum thyroid hormone concentrations (total T3 and T4 and free T3 and T4) and compare these levels with thyroid histomorphology and OC concentrations in serum, liver and blubber.

4. To measure the levels of vitamins A and E in serum, liver and blubber (at multiple depths) and evaluate the relationships between vitamin concentration and gender, season of collection, reproductive status and age class, as well as the associations between vitamin concentrations and percent lipid and concentrations of OC contaminants and selected essential elements.

5. To evaluate the presence and use of serum haptoglobin as an indirect indicator of acute inflammation and health.

6. To investigate and optimize aging techniques (aspartic acid racemization, baleen isotopic $\delta^{13}C$ analysis and pentosidine quantification) in the bowhead whale.

Scope of Study

In this study, blubber indices (quantity and quality), histology, morphometrics, contaminant levels and serum measures are utilized to produce a general health assessment of subsistence-caught bowhead whales. Comparisons are made between whales harvested in spring (departure from the Bering Sea, arrival in the Beaufort Sea) versus the fall (departure from the Beaufort Sea, returning to the Bering Sea), age classes (juveniles/yearlings, subadults, adults) and whales of differing gender and reproductive stages.

Chapters two, three and four deal with biomarkers of health and toxicant exposure. In chapter two, "Thyroid function and histomorphology as biomarkers in bowhead whales (*Balaena mysticetus*)", serum thyroid hormone (TH) concentrations were assayed to evaluate health status or effects of toxicant exposure in marine mammals, including beluga whales (*Delphinapterus leucas*), harbor porpoises, grey seals (*Halichoerus grypus*) and California sea lions (*Zalophus californianus*) (DeGuise *et al.*, 1995; Hall *et al.*, 1998; Debier *et al.*, 2005; Rolland, 2000; Schumacher *et al.*, 1993). Histological sections from thyroid glands of the bowhead whale were examined in conjunction with serological TH analyses. Serum was assayed for total and free triiodothyronine and total and free thyroxine via radioimmunoassay. This was

compared to OC levels in liver and blubber. Histomorphology of thyroid tissue was also assessed via light microscopy and the utilization of an epithelial-follicular index.

Chapter 3, "Vitamin A and E tissue distribution with comparisons to organochlorine concentrations in the serum, blubber and liver of the bowhead whale (*Balaena mysticetus*)", examines Vitamin A and E concentrations in the liver, blubber and serum of subsistence-harvested bowhead whales obtained over a four-year period (1998-2001). Vitamin concentrations were investigated with respect to sex and reproductive status (males, females, pregnant females), age groups, and season of capture. Certain persistent organic contaminants are known to negatively correlate with retinol concentration in serum of pinnipeds and cetaceans (Rolland, 2000; Simms and Ross, 2000). The relationships between serum, liver and blubber retinol and serum and blubber OC concentrations were evaluated in the bowhead whale.

Chapter 4: "Serum haptoglobin detection in bowhead whale (*Balaena mysticetus*) serum" assesses the clinical use of the acute phase protein, haptoglobin, in marine mammal health assessment. A haptoglobin-hemoglobin binding assay was used to analyze serum from 51 bowhead whales for the presence of serum glycoproteins (haptoglobins) that are produced during the acute phase reaction. Haptoglobins are known to increase during extensive tissue damage or necrosis in many terrestrial species. Whether haptoglobins are present at low levels or absent in the serum of healthy individuals may vary by species and had not been previously measured in mysticetes.

Chapter 5 is titled "Renal interstitial fibrosis, pulmonary fibromuscular hyperplasia and other findings from a histological assessment of the bowhead whale (*Balaena mysticetus*)". During the subsistence harvest of bowhead whales, we had the opportunity to collect tissues for histological assessment. We examined formalin-fixed tissue samples via light microscopy from 64 bowhead whales. Our objectives were to describe a range of normal histological findings in the species and to define a background of naturally occurring disease, identifying/discriminating abnormalities that could be attributed to heavy metal/mineral toxicity, specific disease entities or capture. Qualitative observations were made more quantitative through the use of histological measurement and rating profiles, which allowed the assignment of histological observations to a clearly-defined scoring system. Results are interpreted in the

context of "gender groups" (males, females, pregnant females), age groups and season of capture, and a CD of archived histological images from bowhead whale tissues has been produced to accompany this thesis.

Chapter 6, "Heavy metal and mineral concentrations and their relationship to histopathological findings in the bowhead whale (*Balaena mysticetus*)", examines the heavy metal and essential element assessment concentrations in selected tissues of the bowhead whale. Baseline values for this species are established using age (in years), gender and season. An examination of the relationship between heavy metal/mineral concentrations in kidney and liver and histological changes noted as a part of the overall health assessment is conducted.

Blubber is integral to the survival of arctic marine mammals, including large whales, which employ it for a variety of uses. Bowhead whales have an extensive blubber layer, reaching a thickness of up to 50 cm and representing nearly 50% of body mass (Craig George, personal communication). Blubber quantity and quality data are presented in Chapter 7: "The distribution of collagen and its relationship to lipid and blubber thickness in the bowhead whale (*Balaena mysticetus*)". The distribution of blubber thickness at six different sites (five depths at each site) on each whale sampled is described. The percentages of collagen and lipid at each of these sites are investigated and analyzed with respect to gender, age and season of capture.

The two final chapters deal with age estimation of bowhead whales, a measure critical to the proper interpretation of the data presented within this thesis. The first chapter (Chapter 8) "Update on age estimation of bowhead whales (*Balaena mysticetus*) using aspartic acid racemization" evaluates the use of aspartic acid racemization technology to age whales via eye lens nucleus analysis. Ninety-eight eye globes (from 84 individual bowhead whales) were collected and analyzed to estimate ages of the whales using the aspartic acid racemization aging technique. Racemization rate (k_{Asp}) was based on data from earlier studies of humans and fin whales. The D/L ratio at birth $(D/L)_0$ was estimated using eyes from two term bowhead fetuses. Its variance, as well as the variance of the D/L ratios measured for whales older than age 0, was calculated via analysis of variance using multiple aspartic acid measurements from the same whale. Age estimates for each whale and standard errors of these age estimates were obtained using the delta method. Chapter 9, titled "Collagen aging in the bowhead whale (*Balaena mysticetus*)", evaluates the potential of

aging whales via the analysis of a small amount of skin (from a biopsy dart sample or collection at necropsy) via the quantification of pentosidine. Pentosidine, a marker of glycoxidative stress in skin collagen, increases as collagen ages in several terrestrial species that have been studied. Results include the quantification of pentosidine and other collagen related products from the dermal collagen of 47 bowhead whales and comparisons to aspartic acid racemization aging results.

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Chapter Two

Thyroid function and histomorphology as biomarkers in bowhead whales (*Balaena mysticetus*)[1]

2.0 Abstract

Serum thyroid hormone (TH) concentrations have been used alone or with other measurements to assess health status or effects of toxicant exposure in marine mammals. Histological sections from thyroid glands of the bowhead whale (*Balaena mysticetus*) were examined in conjunction with serological TH analyses. Serum was assayed for total and free triiodothyronine and total and free thyroxine via radioimmunoassay. Histomorphology of thyroid tissue was assessed via light microscopy and the utilization of an epithelial-follicular index (EFI).

Age, sex or season did not significantly affect serum TH levels. However, TH concentrations in pregnant/lactating females were found to be significantly lower than other gender groups investigated. The EFI and epithelial height (EH) were greater in spring subadult and adult whales than those landed in the fall. No correlation was found between serum TH concentrations and serum, blubber or liver levels of select polychlorinated biphenyl metabolites and organochlorine congeners examined.

Low variability in concentrations of the serum THs across age, season and gender groups supports the existence of strong homeostatic mechanisms for maintenance of TH concentrations in these presumably healthy animals. Departures from these ranges may indicate a disturbance in these regulatory mechanisms and may be a useful indication of toxicity or other health disorders.

Key words: *Balaena mysticetus*, biomarker, bowhead whale, cetacean, EFI, iodine, organochlorines (OCs), polychlorinated biphenyls (PCBs), thyroxine, triiodothyronine.

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[1] Rosa., C., O'Hara, T.M., Hoekstra, P.F., Refsal, K.F., Blake, J.E. Thyroid function and histomorphology as biomarkers in bowhead whales (*Balaena mysticetus*). Prepared for the Canadian Journal of Zoology

2.1 Introduction

Serum thyroid hormone (TH) levels have been suggested as useful biomarkers of contaminant exposure and as surrogate measures of health in several species of marine and terrestrial mammals (Rolland 2000; Schumacher et al. 1993; DeGuise et al. 1995; Beland et al. 1993). A useful biomarker should reliably measure a change induced by one or more contaminants (or class of compounds) in the biochemical or cellular components of a process, structure or function (NRC 1989). Unlike a diagnostic test used to assess clinical disease, a biomarker must be sensitive enough to detect an early change that may eventually progress to overt clinical disease or significantly altered physiology, such as impaired immune function or reproduction. Most biomarkers do not possess the specificity to identify the particular agent or agents causing the measured change. Instead, they indicate that a change has occurred and that subsequent investigation is warranted; however, a good biomarker should help direct the diagnostic effort. Biomarkers can be separated into three broad groups: biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility (Arkin 1991). The search for indicators of marine mammal health or toxicant exposure has identified many potential candidates, including cytochrome P450 isozyme induction (Angell et al. 2004; Hahn et al. 2000), c-reactive protein (Funke et al. 1997), vitamin A (Simms and Ross 2000), benzo(a)pyrene monooxygenase (BPMO) activity (Fossi et al. 2003) and serum thyroid hormones (Schumacher et al. 1993; Hall et al. 1998, 2003).

Thyroid hormones are relatively easy to measure in most species but reliable results depend on method validation. There is little published information available on cetacean serum TH levels and thyroid gland histomorphology. Nor are there many references describing the gross anatomical features of cetacean thyroid glands (Harrison 1969; Ridgeway and Patton 1971; Shimokawa 2002). With few exceptions, mammalian thyroid histomorphology and ultrastructure exhibit such little variability that it would be difficult to differentiate between species using light or electron microscopy. In contrast to microscopic and ultrastructural anatomy, total and free serum TH reference ranges vary significantly from one mammalian species to another (Joasoo et al. 1975; Tomasi et al. 1998; Ortiz et al. 2000). Within a single species, many different factors such as season, nutritional status, reproductive state, contaminant load and health condition

can influence thyroid hormone activity (Bhagavan, 2002; Rolland 2000; Schumacher et al. 1993; DeGuise et al. 1995; Beland et al. 1993).

In cetaceans, correlation of thyroid hormone concentration variability with season may be influenced by factors such as water temperature, day length, reproduction/lactation, and feeding/fasting (St. Aubin and Geraci 1988; St. Aubin and Geraci 1989; St. Aubin et al. 2001). Substantial variability in the function and histomorphology of thyroid glands that correlates with seasonal changes was first identified in beluga whales (*Delphinapterus leucas*) (St. Aubin and Geraci 1989; St. Aubin et al. 2001).

Thyroid hormones are important in the hormonal regulation of seasonal breeders (Dahl et al. 1994; Viguié et al. 1999). Sex-related differences in TH concentration have been found in marine mammals, though results vary. Age and sex-related differences in T4 and T3 have been found in the beluga whale (St. Aubin et al. 2001), while no effect of sex on T4, T3 or thyroid hormone binding capacity (THBC) was found in bottlenose dolphins (*Tursiops truncatus*) as measured by radioimmunoassay (Greenwood 1979). There are limited data on thyroid hormone levels in pregnant wildlife and no published information for pregnant cetaceans found in the scientific literature.

Alterations in thyroid function and morphology have been induced by PCBs in experimental animals (Bryne *et al.* 1987; Brouwer 1989) and have been related to the effects of organochlorines (OCs) on TH transport proteins, receptors, and TH activity. Captive harbor seals (*Phoca vitulina*) experimentally fed fish contaminated with PCBs exhibited reductions in serum tT4 and fT4 concentrations as compared to seals that ingested fish that were from non-contaminated waters (Brouwer et al. 1989). Plasma T3 deficiency has also been associated with chlorinated hydrocarbon exposure in spotted seals (*Phoca largha*) and ribbon seals (*Phoca fasciata*) (Chiba et al. 2001). These findings are important to consider in the evaluation of marine mammal population health.

The bowhead whale (*Balaena mysticetus*) is an endangered mysticete species that lives in arctic and sub-arctic waters. Although the Bering-Chukchi-Beaufort Sea (BCBS) Stock was commercially exploited to near extinction in the late 1800's, it is currently growing (George et al., 2004) and is an important subsistence species to residents of northern Alaska, Russia and Canada. The BCBS stock undertakes a yearly migration between the Bering Sea region and the Beaufort Sea to reach the highly productive

summer feeding grounds located there, providing the opportunity to obtain high quality biological samples from landed whales in the spring and fall.

As part of a larger health assessment study, we analyzed serum thyroid hormone concentrations (total T3 and T4 and free T3 and T4) in bowhead whales and compared these levels with their thyroid histomorphology. Our primary objective was to describe the relationship between thyroid function and histomorphology with consideration given to season, gender/reproductive stage, age and OC concentrations in serum, blubber, and liver. As a secondary objective, we describe the gross anatomy of the thyroid gland in the bowhead whale.

2.2 Materials and Methods

2.2.1 Sample collection/processing

Thyroid tissue and blood samples were collected during the 1998-2002 spring and fall Inuit subsistence harvest in Barrow, Alaska. These sample collections were conducted with permission of the Barrow Whaling Captain's Association and the Alaska Eskimo Whaling Commission (AEWC) through the Department of Wildlife Management (North Slope Borough, Alaska) under the purview of a National Oceanic and Atmospheric Administration (NOAA) permit [#932-1489-00 and 932-1489-03 for the Marine Mammal Health and Stranding Program (MMHSRP) program issued to Dr. Teri Rowles]. Blood was collected approximately 2-14 hours post-mortem from the palatal sinus of 61 bowhead whales and one fetus into untreated evacuated red top tubes (Vacutainer/BD, New Jersey USA). The blood was allowed to clot and was centrifuged for 10 minutes at 3500 g within 4-6 hours of collection. The serum was transferred by pipette to a 5ml plastic culture tube and frozen at -20°C then archived at –80°C until thawed for hormone analyses.

A central portion of the thyroid gland (n=24) was preserved in 10% buffered formalin within 3-14 hours of death. After fixation, 5 μ paraffin embedded sections were stained with hematoxylin and eosin (H&E). Digital photomicrographs and measurements were taken using a Zeiss Axiocam camera and Axiovision software (version 3.2, Carl Zeiss, Inc., One Zeiss Drive, Thornwood, NY USA).

2.2.2 Hormone analyses

All serum analyses were performed at the Diagnostic Center for Population and Animal Health, Michigan State University, East Lansing, Michigan (USA). Total thyroxine (T4) was measured with a commercially available solid-phase radioimmunoassay (RIA) kit (Clinical Assays Gammacoat M Total T4 ^{125}I RIA Kit; DiaSorin Inc, Stillwater, Minnesota USA). Specificity data from the manufacturer identified 92% cross-reactivity with D-thyroxine, 2.1% cross-reactivity with D- and L-triiodothyronine (T3), and less than 0.1% cross-reactivity with other iodothyronines. The reagents were prepared as described in the manufacturer's protocol. Modifications were made to the protocol to enhance the analytical sensitivity of the assay. The volume of sample or standard was increased from 10 to 25 µl for all samples analyzed. A "lower" standard was made by mixing equal volumes of 0- and 13-nmol/L standards. The high standard (257 nmol/L) included in the kit was discarded, leaving the 156-nmol/L standard as the highest in the curve. After pipetting the sample or standard and 1 mL of radioligand solution into antibody-coated tubes, the assay was incubated at room temperature (~22°C) for 3 hours. The sensitivity assay, defined as the concentration of T4 at 90% specific binding was 3 nmol/L (data from 10 assays). When l-thyroxine was added to aliquots of a pool of bowhead whale serum to create increases of 26 and 52 nmol/L, 93% and 81% of added T4 was measured in the respective assays. A pool of bowhead whale serum, with a concentration of T4 of 94 nmol/L, was diluted with "0" standard at 50% and 25% dilutions. Assay of these diluted samples yielded 91% and 99% recoveries, respectively, when corrected for dilution. Intraassay repeatability was determined in a pool of bowhead whale serum with a 94 nmol/L concentration of T4. The respective intraassay and interassay coefficients of variation (CV) for 10 replicates of this pool were 0.038 and 0.024. Total triiodothyronine (T3) was measured by a charcoal separation RIA whose procedures and modifications have been previously described in Refsal et al. (1984) and Panciera et al. (1990). The sensitivity of the assay, described as the concentration of T3 at 90% specific binding was 0.26 nmol/L (data from 10 assays). When T3 was added to aliquots of a pool of bowhead whale serum to produce increases of 1.5 and 3.0 nmol/L, 113% and 114% of added T3 was measured in the assay, respectively. A pool of bowhead whale serum with a T3 concentration of 1.03 nmol/L was diluted to 50% and 25% dilutions in "0" standard, with 107% and 158% recoveries, respectively, when corrected for dilution. Assay repeatability

was determined with the same pool of bowhead whale serum. The respective intra- and inter-assay CV for 10 replicates of this pool were 0.091 and 0.097.

Assays for free T4 in bowhead whale sera were done using materials and reagents in a commercially available kit (Free T4 by equilibrium dialysis; Nichols Institute Diagnostics, San Clemente, California USA). The procedures for equilibrium dialysis and RIA of T4 in dialysate were done following the manufacturer's protocol. The manufacturer reported less than 0.044% cross-reactivity of other iodothyronines in the RIA. The sensitivity of the assay, defined as the concentration of free T4 at 90% specific binding, was 1.8 pmol/L (mean of 10 assays). Estimates of dilutional parallelism and recovery were made with dialysates of bowhead whale serum. When a pool of dialysate with a concentration of free T4 of 29 pmol/L was diluted with dialysate buffer by 50% and 25%, 100% and 158% of expected amounts of free T4 were measured in the assay, respectively. When aliquots of T4 equivalent to 5, 12, 29 and 66 pmol/L were added to the same pool of bowhead whale dialysate, 104%, 102%, 113%, and 108% of the respective added T4 was measured in the assay. Estimates of repeatability were also made with the same pool of bowhead whale serum. For 10 replicates of each pool, the respective intra- and inter-assay CV's were 0.089 and 0.148, respectively.

Free T3 was measured using a commercially available solid-phase RIA based on competition of endogenous free T3 with a ^{125}I-labeled triiodothyronine derivative (Clinical Assays GammaCoat Free T3 ^{125}I RIA Kit; DiaSorin Inc, Stillwater, Minnesota, USA). The kit protocol from the manufacturer described 100% antibody cross-reactivity with l-triiodothyronine and less than 0.2% cross-reactivity with all other iodothyronines tested. The volumes of samples, standards, and radioligand were used according to the manufacturer's protocol. A modification to the assay procedure was to extend the duration of incubation from 90 minutes to 3 hours in a 37°C waterbath. This change was done to assure equilibration of maximal binding for assay runs that consisted of a standard curve and 53 samples. The sensitivity of the assay, as defined as the concentration of free T3 at 90% specific binding, was 1.2 pmol/L (based on data from 10 assays). In analog-based RIA for free T3, there are multiple binding interactions between the endogenous hormone, the T3-derivative, assay antibody, and endogenous binding proteins. In this circumstance, assessment of dilutional parallelism and recovery is not possible. For a bowhead whale serum pool with a

free T3 concentration of 2.8 pmol/L the respective intra- and inter-assay CV's were 0.044 and 0.089 (10 replicates), respectively.

2.2.3 Histomorphometric analysis

An epithelium-follicular index (EFI) was calculated using a technique adapted from Sidor (1971) using digital images (n=24 whales). One hundred thyroid follicles per whale were selected at random for measurement using a labeled grid (Lovin Field Finder, Gurley Precision Instruments, Troy, New York USA). Samples showing evidence of extensive autolysis resulting in cell or follicle distortion were not measured. A follicular length (FL) was taken between the farthest distant two points in the follicle. A follicular width (FW) was then measured perpendicular to the initial FL measurement. Four measurements of epithelial height (EH) per follicle were then made at each of these four extremities (from the innermost aspect to the outermost aspect of the follicle lining cell). The following formula was used to calculate follicle size (FS):

$$FS = \frac{FL + FW}{2}$$

Once FS was calculated, the epithelium-follicular index was determined:

$$EFI = \frac{EHx100}{FS}$$

2.2.4 Aging

Whales in this study were designated juvenile, subadult, or adult (Table 2.1). Baleen stable isotope analysis of carbon ($\delta^{13}C$) signature and aspartic acid racemization of eye lens nuclei were used to determine ages of 35 whales (George et al. 1999; Rosa et al. 2004; Lubetkin et al. 2004). The remaining 26 whales were categorized using a combination of body length, baleen length and gonadal size/development (George et al. 1999; O'Hara et al. 2002; Tarpley and Hillmann 1999).

2.2.5 Persistent organochlorine (OC) analyses

The OC dataset used in this study was previously reported (Hoekstra et al. 2002; 2003).

Many OCs are known to have a relatively high affinity for the T4 receptors on transthyretin (TTR) or impair thyroid function (Ishihara et al. 2002). Potential relationships/interactions between concentrations of various OCs, including forty PCB congeners and selected hydroxylated (OH-PCB) and methylsulfone-containing (MeSO2-PCB) PCB metabolites occurring in greatest average concentrations in serum, blubber and liver were selected for multivariate analysis (Appendix 2.7). These OCs were detected at relatively low levels in most whales.

2.2.6 Statistical analysis

Data are presented as the mean and standard deviation (SD). Thyroid data were analyzed by a three-way analysis of variance (GLM, general linear model) and student's T-test (EFI and follicle size comparisons). Multivariate methods (canonical correlation) were used to determine thyroid hormone/OC relationships. Wilks's lambda was used to test the significance of the first canonical correlation and a likelihood ratio test was used to test the linear relationship between the canonical variables.

All statistical analyses were performed on the SAS operating system (SAS Institute, Inc., SAS Campus Drive, Cary, NC USA). Values were considered significantly different at $P<0.05$.

2.3 Results

The bowhead whale thyroid gland is located along the ventral trachea, cranial to where the trachea bifurcates into primary bronchi. Right and left lobes are easily discriminated and no isthmus was observed between the two lobes. The histological appearance of the thyroid in the bowhead whale was similar to other mammalian species, with follicles of variable size lined by simple epithelium of variable height. Qualitatively, thyroid follicles from the thyroid glands of bowhead whales landed in the spring tended to be small with a diminished amount of colloid and tall-cuboidal to columnar epithelial lining cells with large, open-faced nuclei. The typical appearance of thyroid glands collected from adults and subadults in the fall was characterized by colloid-distended follicles lined with attenuated epithelial cells.

The serum thyroid hormone results are summarized in Table 2.2. There was no significant difference in thyroid hormone levels found between seasons, sexes or among age (length) groups.

However, pregnant and lactating females (n=5) had significantly lower tT4 and fT4 than non-pregnant adult females (n=7) and other age-sex classes (n=49) (Table 2.3). Data from one fetal serum sample (not included in this data set) indicate that near-term fetal thyroid levels (tT4 = 136 nmol/L, tT3 = 3.1 nmol/L, fT4 = 53 pmol/L, fT3 = 9.8 pmol/L) are high in comparison to the averages for juvenile, subadult and adult whales.

Sub-adult and adult follicle size was significantly larger in fall versus spring (Fall: $\bar{x}$=307.56 ± 190.73, Spring: $\bar{x}$=67.68 ± 18.15, P=0.036). There was no difference between the sexes in any age group. Figure 2.1 depicts thyroid follicle size frequency distributions (expressed as %'s) in spring and fall. Figure 2.1 (A) depicts the distribution of various follicle size classes including all age groups in the spring season. Figure 2.1 (B) depicts this distribution including all age groups of whales in the fall season. When compared to Figure 2.1 (A), there is no significant difference found between spring and fall distribution (P= 0.343). Figure 2.1(C) represents fall juvenile distribution only, which is statistically similar to the spring distribution of all age groups (P=0.062) Figure 2.1(D) shows a follicle size distribution with the juvenile whales (which exhibit active follicular histology/EFI in fall) omitted from the fall graph. The fall distribution in figure 2.1(D) (subadults and adults only) is significantly different from the spring distribution in Figure 2.1(A), with more of the larger follicle size classes represented (P=0.0004). A photomicrographic comparison of spring and fall thyroid histology is shown in Figure 2.2. There were no significant differences found in EFI by three-way ANOVA when all age classes were analyzed together (P=0.65). For subadult and adult whales (juveniles omitted due to their maintenance of active epithelium in spring and fall), those harvested in the spring have significantly higher EFI than those harvested in the fall (Table 2.4).

The follicular epithelium in juvenile whales, irrespective of harvest season, had tall cuboidal to columnar epithelial cells lining follicles (Table 2.4). In the fall, there was a significant difference in EFI between juvenile whales and subadult/adult age classes.

There was no correlation between serum TH concentration and any of the serum, blubber and liver OC metabolites and PCB congeners investigated (all P values < 0.05).

2.4 Discussion

Serum TH concentrations in bowhead whales were consistent over age classes (juvenile, subadult and adult) and gender/reproductive groups with pregnant females being the exception. The significantly lower serum TH concentration found in pregnant/lactating females is of interest, especially with respect to biomarker and health assessment interpretation. There is considerable variability in thyroid hormone levels among mammalian species during pregnancy (Kimura et al. 1990; Calvo et al. 1990; Tomasi et al. 1998; Fantz et al. 1999). The fetal brain is known to be highly sensitive to deficient or excessive levels of TH (Porterfield 1994). The iodine-rich diet of the bowhead whale is a likely determinant of the differences we observed in pregnant females, with the lowered maternal levels possibly serving to protect the fetus from high circulating iodine levels. In this study, the pregnant/lactating reproductive group consisted of five females, thus, it would be best to reevaluate these findings with a larger number of samples representing additional stages of pregnancy. The single fetal serum sample indicates that near-term fetal TH levels were high in comparison to the averages for non-neonates, which is similar to ruminants and horses, where circulating concentrations of thyroid hormones are much higher in neonates than adults (Irvine and Evans 1975; Refsal et al. 1984).

No seasonal differences (fall versus spring) were found in the serum levels of TH in landed bowhead whales: however we observed marked seasonal differences in the thyroid histology. Beluga whales exhibited seasonal changes in TH (St. Aubin and Geraci 1989). St. Aubin and Geraci (1989) suggested that this observed seasonal correlation relates to increased water temperature of the (inland) estuaries that belugas seasonally occupy. However, no such effect of water temperature was observed in studies of captive *Tursiops* spp. (St. Aubin et al. 1996), indicating these changes could be species-specific. Thyroid hormone concentration and histology were also examined in the beluga whale (*Delphinapterus leucas*) during different phases of their migration. Higher levels of circulating T3 and T4 were found in the summer months. This difference was accompanied by histological transformation of the thyroid follicular lining cells from a cuboidal to a columnar morphology (St. Aubin et al. 1989). In photoperiod studies of non-cetacean mammals, light restriction has been found to increase the activity of the pineal gland resulting in increased melatonin production (Haldar et al. 1992; Bhagavan 2002). The neurohormonal properties of

melatonin may affect the pituitary, hypothalamus or thyroid hormone metabolism directly in laboratory animals. Melatonin has been reported to decrease blood T4 levels in both rats and hamsters (Haldar et al. 1992; Vriend 1983; Lewinkski et al. 2002; Singh et al. 1969). However, harp seal studies did not indicate a stimulatory action of melatonin in the peripheral conversion of T4 to T3 (Stokkan et al. 1995). The effect of shortened day length on the histology of the thyroid in bowhead whales cannot be discounted, as the cell and glandular morphology suggests that the thyroid gland is substantially less active in fall versus spring. Assessment of photoperiod, including the measurement of serum melatonin, should be considered for future studies.

Intake of iodine from seasonal foraging may be an important factor in triggering the seasonal changes observed in follicular diameter and the height of epithelial lining cells. In the spring, bowhead whales are thought to be at the end of a period of winter fasting (Lowry, 1993). Their prey is iodine rich (Krinsky, 1965; Kon and Thompson, 1970) and is seasonally available in large amounts (summer and fall) (Burns et al. 1993). Whales that migrate from the Bering Sea to the Beaufort Sea region in spring are thought to have been fasting over winter, with intermittent feeding during spring migration as the productivity of the area increases over the summer months (Burns et al. 1993). In the normal mammalian thyroid gland, the limiting step of thyroid hormone synthesis is the uptake of iodide (Bhagavan 2002; Pineda and Dooley 2003). Iodine levels are high in the blubber of marine mammals compared to adipose tissue of terrestrial mammals (Ackman et al. 1975) making this tissue an important storage depot for this element. The colloid-dilated follicles noted in the fall indicate storage is underway. The large, intensive intake of iodine in summer and fall feeding could lead to an adaptive decrease in thyroid hormone synthesis known as the Wolff-Chaikoff effect (Wolff and Chaikoff 1948; Wolff 1969). This phenomenon involves the blockade of iodide organification and thyroid hormone synthesis triggered by high intrathyroidal iodide, which could potentially lead to changes in the histological appearance of the gland. In this scenario, in mid-summer and early fall, the thyroid initially becomes more active under the influence of increased dietary iodine (a period absent from our sampling) and other nutrients. When iodine levels reach a threshold of excess, this block occurs, effectively stopping the hydrolysis of T3 and T4 from thyroglobulin, resulting in colloid-dilated, inactive follicles characteristic of the histology noted in the fall for subadult and adult bowheads. Humans

and laboratory rodents subsequently experience what has been termed an "escape phenomena" from the Wolff-Chaikoff block 2-8 weeks after the initiation of exposure to high levels of iodine. This phenomenon allows serum thyroid levels to return to normal due to inhibition of the symporter that introduces iodine into the cell. This causes a decrease of intracellular iodine which removes the block in thyroid peroxidase expression which eventually leads to an increase of T4 and T3, sparing the organism from hypothyroidism in the presence of chronically increased levels of iodine (Wolff and Chaikoff 1948; Wolff 1969). This block provides a potential explanation for the normal serum TH concentrations noted in the presence of inactive thyroid histology and decreased EFI in fall.

Juvenile whales maintained an active thyroid histologic appearance in both spring and fall. A relationship may exist between the persistence of this active epithelium and the rapid growth phase experienced during this phase of life (although serum hormone levels do not reflect higher circulating TH levels). It is possible that this age class may convert, bind or excrete TH in a different manner than subadult and adult whales. This difference could also be related to less efficient feeding mechanisms in these juveniles due to shorter baleen and inexperience in feeding methods and sites. In this case, iodine exposure may be inadequate to induce the Wolff-Chaikoff block/escape allowing for maintenance of a histologically active thyroid. Measurements of circulating and stored iodine levels are needed to further investigate these differences.

Published research provides compelling evidence that some OCs, including several parent PCBs and metabolites, alter the thyroid axis. Alterations in thyroid function and morphology have been induced by PCBs in laboratory and marine mammals (Bryne *et al.* 1987; Brouwer 1989; Skaare et al. 2001; Debier et al. 2005) and have been related to the interactions of OCs with thyroid hormone transport proteins. Hydroxylated OCs and other halogenated phenolic compounds are selectively retained in blood due to their structural similarities to T4 and binding affinity with transthyretin (due to similar chemical configuration at the OH- binding site and surrounding halogen substitution at the vicinal C-atoms) (Ishihara et al. 2002). Transthyretin (TTR) functions in the transport of both retinol and thyroid hormone and accounts for ~20% of circulating T4 in experimental animals. Many OCs are similar in structure to TH and retinol. Often the affinity of the transport protein for these contaminants is far greater than for TH (Ishihara et al. 2002; AMAP Assessment 1998; AMAP Assessment 2002). Bowhead whale serum, blubber and liver OC

concentrations were low in comparison to other Arctic marine mammals, and were of a magnitude lower than those seen in large mysticete whales in the Northern Atlantic Ocean (Hoekstra et al. 2002). This is likely attributable to the low trophic level on which the bowhead whale feeds and differences in OC deposition between eastern and western Arctic regions (Wagemann et al. 1990; 1996; Hoekstra et al., 2002a; 2002b). Measurement of TTR concentrations would be useful to make a more complete evaluation of TH binding dynamics in bowhead whales. It is possible that OC concentrations of the magnitude noted in this study are not sufficient to produce a measurable effect on TH or retinol. This may support the use of TH as a biomarker, as subclinical or clinical effects of these contaminants have not been seen in this stock. However, it is also possible that circulating TH levels in bowhead whales may not be an appropriate biomarker for low-dose exposure to OCs. In cases such as this, other biomarkers may be more appropriate. For example, hepatic type I monodeiodinase (MDI) is an enzyme involved in TH homeostasis. Research in other species has shown reduction in MDI activity to be a more sensitive indicator of PCB exposure (Gould et al. 1999). Hepatic type I monodeiodinase may have potential as an alternative biomarker in cases of low dose exposure to OCs, though it has yet to be investigated in marine mammals.

The above data indicate some of the confounding variables encountered when using serum TH concentrations and thyroid histology as biomarkers. There are also issues of validity and reliability of measurements to be considered as well as capacity to interpret "impact" without assay validation and a sense of "normal".

A degree of agreement must exist between the biomarker and the process it is measuring for it to be valid. In this case, we have low concentrations of OCs of interest measured (as compared to other related mysticetes and marine mammals of the region) and a population experiencing positive growth that possess an arguably surprising lack of pathology (gross or histological) (George et al., 2004; Rosa, unpublished data). A lack of controlled experimental trials hinders full understanding of the response of TH to stressors in marine mammals, but this alone should not disqualify its utility as a biomarker. Most information on TH in marine mammals has been gathered retrospectively from wild populations, and data from rodent models are supportive of the theory of TH suppression in cases of exposure to certain levels of OCs. Biomarker levels may undergo modifications in relation to the hormonal status, age and sex of an organism; this limit

can be minimized if the reproductive cycle, physiology and variations between individuals of the species in question are known. Our results have found levels of serum TH to be remarkably stable over age classes, seasons and between sexes. Age and seasonally related changes were noted in the histology and EFI, but a robust control of serum hormone levels appears to occur in the presence of these obvious changes. This may be attributable to homeostatic mechanisms that maintain serum levels in a normal range when intake of food (iodine) is marginal or excessive and may indicate that deviation from the norm is unlikely in apparently "healthy" individuals with low OC burdens. This lends greater credibility to the hypothesis that normal TH levels are maintained in this state and deviations from the norm are likely to be caused by something other than differences found over age classes, seasons and between sexes.

The accuracy of a given biomarker needs to be determined and reproducibility is important for it to be considered reliable. In fieldwork, this is difficult to realize. The subjects are wild, and their health and the contaminants that they are exposed to cannot be controlled. Even in captive marine mammals, subject numbers are low and the lack of ethical acceptance for clinical toxicological trials hinders full understanding of the behavior of many substances of interest. Multiple measurements of a biomarker from several animals with similar levels of a given contaminant are likely to differ. We have attempted to minimize this concern by gathering a large sample size of animals of two genders, in two seasons and over different age groups. However, questions remain as to how specific the effects of OCs are for TH concentrations and with what amount of accuracy serum TH represents the total body OC burden. This study is an example of more complete types of data collected alongside the limitation of dealing with larger cetacean species not amenable to captive research.

Previous TH investigations in marine mammals have produced variable results. Putatively, no correlation was found between the low concentrations of OC congeners and serum TH levels in this healthy population of whales, and little variability in serum TH concentrations was detected over age/season/gender groups (with pregnant females excepted). These findings further support the use of TH concentration as a potentially reliable marker of OC exposure. However, the dynamics of thyroid hormone production in fasting-adapted cetaceans warrants continued research. At present, we recommend the use of TH as a biomarker in conjunction with other endpoints that provide insight into the health and contaminant

exposure of the individual. Results should be interpreted with caution. These data provide valuable information from a rare opportunity to collect samples from a large number of whales in good health, sampled across these strata. Future work involving cetacean thyroid hormones and histology should be able to build on this to formulate new hypotheses regarding TH concentrations in this population and populations of other large mysticetes. It is important for additional data to be gathered, as this will add to our knowledge of TH dynamics and the value of these hormones as biomarkers, not only of contaminants, but of ongoing (offshore industrial activities) and emerging (climate change) potential stressors.

2.5 Acknowledgements

We express our appreciation to the Department of Wildlife Management, North Slope Borough (Barrow, Alaska), the Cooperative Institute for Arctic Research (CIFAR), the Alaska IDeA Networks for Biomedical Research Excellence (INBRE) and NOAA for generous funding and logistical support. We also thank the Inupiat subsistence whalers in Barrow and Kaktovik (Alaska USA) and the Alaska Eskimo Whaling Commission (AEWC) for graciously allowing us to access these samples. Individual thanks to Victoria Woshner, Lara Dehn, Ron Barry, Dana Thomas, Mike Castellini, Doug DeMaster, Cyd Hans, Craig George, Benny Akootchook, Chris Terzi, Peter Graham and Susan Beyerlein for their help with the manuscript, tissue collection and analytical support. Bowhead whale samples were collected under the authority of permit #932-1489-00 for the MMHSRP program (Dr. Teri Rowles). Support for this project came from CIFAR/NOAA (NSB BU3613) and Alaska INBRE grant number 2 P20 RR016466 from the National Center for Research Resources, National Institutes of Health, Dept. of Health.

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Table 2.1.
Bowhead whale age group definitions as determined via aspartic acid racemization, baleen stable isotopic measurement ($\delta^{13}C$) and histological analyses.

Juvenile:	1-3 year old whales that experience an accelerated period of growth
Subadult:	3 years of age to approximately 22 years of age in males and 25 years of age in females (sexually immature)
Adult:	greater than 22 years of age in males and 25 years of age in females (sexually mature)

(George et al., 2001, 2004, Lubetkin et al. 2004, O'Hara et al., 2002, Rosa et al., 2004)

Table 2.2.
Thyroid hormone (tT4, tT3, fT4 and fT3[1]) concentrations in the bowhead whale. Mean ($\bar{x}$), standard deviation (SD), range and number sampled (n) listed for (A) All whales sampled in the BCBS stock (excluding pregnant females) and (B) pregnant female bowhead whales only.

A.

Hormone	n	units	$\bar{x}$	SD	Range
tT4	58	nmol/L	83.30	20.5	(40.0 - 128.0)
tT3	56	nmol/L	1.14	0.42	(0.10 - 2.20)
fT4	58	pmol/L	25.80	7.63	(12.0 - 50.0)
fT3	54	pmol/L	4.22	1.63	(1.50 - 10.40)

B.

Hormone	n	units	$\bar{x}$	SD	Range
tT4	5	nmol/L	59.00*	23.00	(38.0 - 96.0)
tT3	5	nmol/L	1.04	0.29	(0.80 -1.50)
fT4	5	pmol/L	16.80*	6.34	(9.00 - 23.0)
fT3	5	pmol/L	3.18	0.57	(2.40 - 4.0)

[1] tT4: total thyroxine, tT3: total triiodothyronine, fT4: free thyroxine, fT3: free triiodothyronine.
*denotes a significant difference from non-pregnant adult females at $P < 0.05$

Table 2.3.
Thyroid hormone (tT4, tT3, fT4 and fT3[1]) concentrations by age group and season (pregnant females excluded) in the bowhead whale. Mean ($\bar{x}$), standard deviation (SD), and number sampled (n).

Hormone	Age Group	Spring			Fall		
		n	$\bar{x}$	SD	n	$\bar{x}$	SD
tT4	Juvenile	10	79.6	21.6	19	86.4	19.0
(nmol/L)	Subadult	3	92.0	26.5	12	75.0	23.2
	Adult	6	85.7	20.7	8	88.1	22.7
tT3	Juvenile	10	1.22	0.37	17	1.08	0.33
(nmol/L)	Subadult	3	1.00	0.20	12	1.03	0.46
	Adult	6	1.08	0.28	8	1.45	0.63
fT4	Juvenile	10	29.4	7.63	19	26.6	5.23
(pmol/L)	Subadult	3	27.7	2.89	12	21.8	7.57
	Adult	6	23.0	7.95	8	26.9	11.6
fT3	Juvenile	10	4.97	2.85	16	4.09	1.05
(pmol/L)	Subadult	3	3.40	0.58	11	3.59	1.49
	Adult	6	4.20	0.99	8	4.73	1.08

[1] tT4: total thyroxine, tT3: total triiodothyronine, fT4: free thyroxine, fT3: free triiodothyronine.
*There was no significant difference noted in any of the thyroid hormones measured between seasons, sexes or age (length) groups.

Table 2.4.
Epithelium-follicular index (EFI) measurement data in the bowhead whale. (A) All whale age classes sampled. No significant difference was found between spring and fall seasons. (B) Subadult and adult whales sampled only. A significant difference was found between spring and fall seasons. (C) Juvenile whales sampled only. No significant difference was found between spring and fall seasons.

A.

EFI	SD	n	season	*P* value
6.43	1.88	8	Spring	0.665
5.29	0.92	16	Fall	

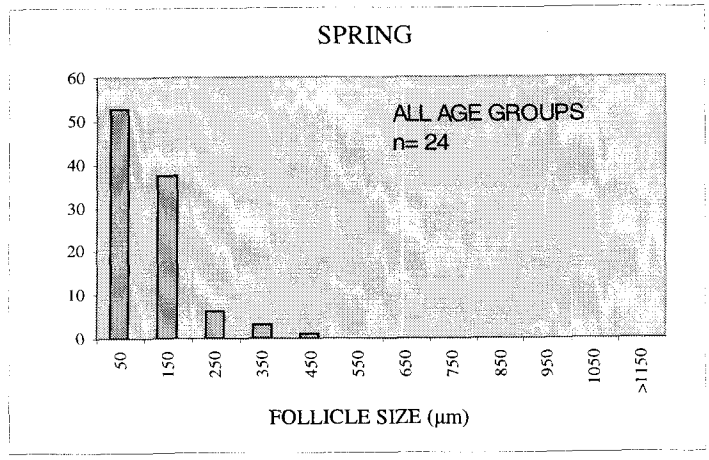
B.

EFI	SD	n	season	*P* value
6.47	1.05	5	Spring	0.0016
4.10	0.74	9	Fall	

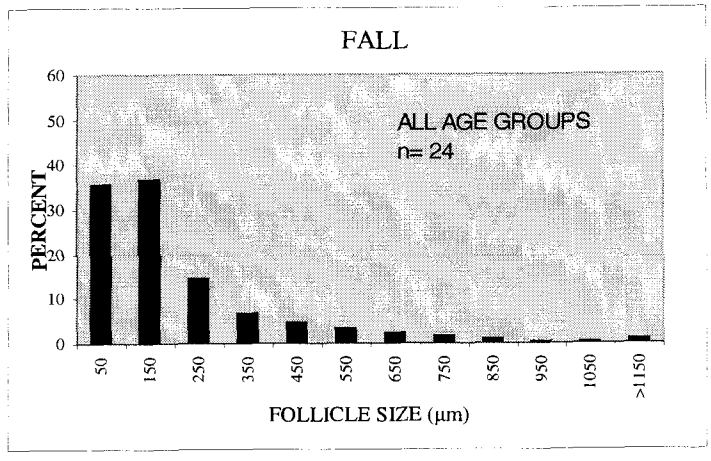
C.

EFI	SD	n	season	*P* value
6.35	0.58	3	Spring	0.655
6.82	1.66	7	Fall	

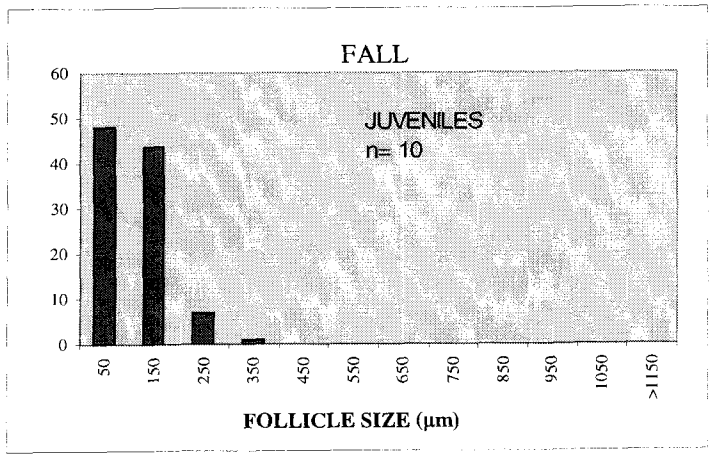
A.



B.



C.



D.

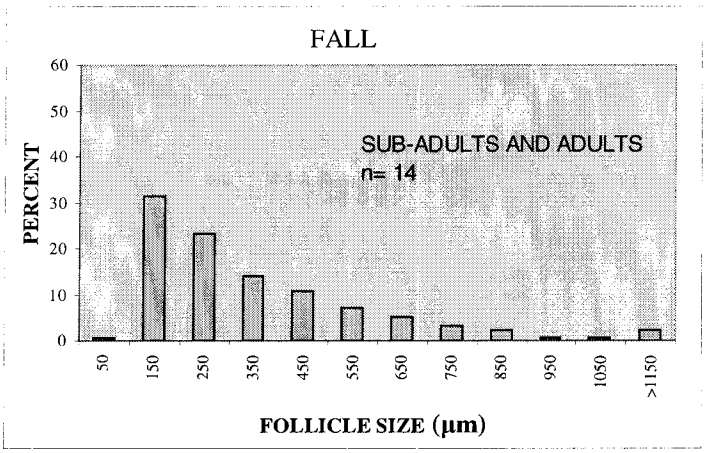
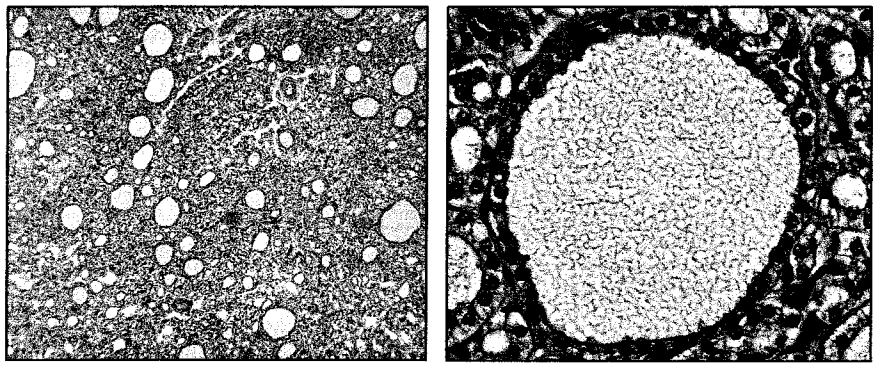


Figure 2.1.
Thyroid follicle size in the bowhead whale (A) Spring thyroid follicle mean percent distribution with all age classes (juvenile, subadult and adult) represented. (B) Fall thyroid follicle mean percent distribution with all age classes (juvenile, subadult and adult) represented. (C) Fall thyroid follicle mean percent distribution with juvenile age class whales represented (subadults and adults omitted). (D) Fall thyroid follicle mean percent distribution with subadults and adult age class whales represented (juveniles omitted).

A.

Spring 40x

Spring 400x



B.

Fall 40x

Fall 400x

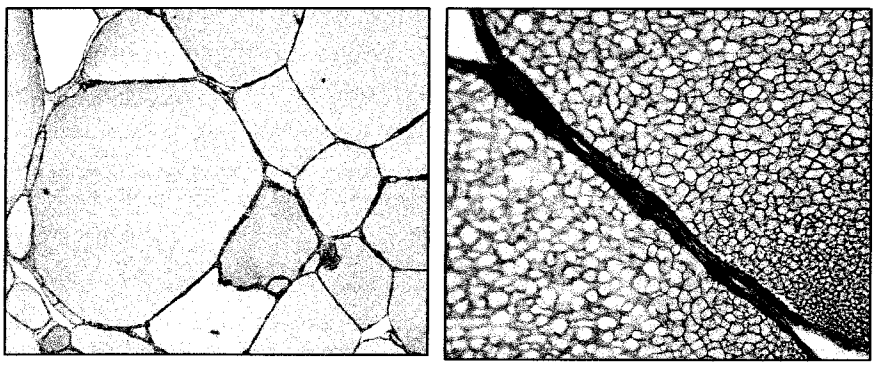


Figure 2.2.
Histological appearance of the thyroid gland in the bowhead whale by season. (A) Spring histological appearance of thyroid follicles. Note the numerous, small, active follicles present in the gland lined with cuboidal epithelial cells. (B) Fall histological appearance of thyroid follicles. Note the large, distended, follicles lined by flat, attennuated epithelium.

2.7 Appendix

List of organochlorine congeners and PCB metabolites (liver, blubber and serum) analyzed by cannonical correlation analyses in the bowhead whale.

op-DDD
ppDDE
a-HCH
HCB
b-HCH
Oxychlordane
cis-Heptachlorepoxide
Dieldrin
pp-DDD
Trans-Nonachlor
g-Chlordane
op-DDT
1-4-DCB
c95&c66
g-HCH(Lindane)
c52c49
pp-DDT
op-DDE
c101
Methoxyclor
c92&c84
Cis-Nonachlor
c82&c151
c119
c44
c163
c110
c153
c31&c28
a-Chlordane
c99
Endrin
c74
c43
c87
c47&c48
c85
c70
c97
d-HCH
c174
c33&c20&c53
c132

c41&c71
a-Endosulfan
c18
c16
c91
c141&c179
Mirex
c105
c15&c17
c146
c64
c56&c60
c83
c136

Chapter Three

Vitamin A and E tissue distribution with comparisons to organochlorine concentrations in the serum, blubber and liver of the bowhead whale (*Balaena mysticetus*)[2]

3.0 Abstract

Vitamin A and E concentrations were determined in liver (n=51), blubber (n=23) and serum (n=53) of subsistence-hunted bowhead whales (*Balaena mysticetus*), between 1998 and 2001. Retinol and alpha-tocopherol were the major forms of vitamins A and E detected, respectively. Liver contained the highest mean concentrations of vitamin A, followed by epidermis, blubber, and serum. Liver also contained the highest mean concentration of vitamin E, followed by serum, epidermis, and blubber. Stratification of retinol and tocopherol was examined throughout the blubber cores collected. Retinol concentrations were significantly higher in the epidermis than in the deeper blubber layers. Tocopherol concentrations were similar for epidermis and the intermediate layer of blubber. Both the epidermis and the intermediate layer of blubber had significantly higher tocopherol concentrations than the innermost and outermost blubber layers. Vitamin A and E concentrations were investigated with respect to gender and reproductive status of females (males, non-pregnant females, pregnant females), age groups and season of harvest. Certain persistent organic contaminants are known to have a negative effect on retinol concentration in serum of pinnipeds and cetaceans. Bowhead whales have relatively low concentrations of organochlorines (OCs) in comparison to other mysticete species. The relationships between serum, liver and blubber retinol and serum and blubber OC concentrations were examined with no significant correlations noted.

Keywords: alpha-tocopherol, Balaena mysticetus, biomarker, bowhead whale, cetacean, organochlorine, retinol, vitamin A, vitamin E

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[2] Rosa, C., Blake, J.E., Mazzaro, L., Hoekstra, P., Ylitalo, G.M., O'Hara, T.M. Vitamin A and E tissue distribution with comparisons to organochlorine concentrations in the serum, blubber and liver of the bowhead whale (*Balaena mysticetus*). Prepared for submission to Comparative Biochemistry and Physiology Part A

3.1 Introduction

Vitamin A is necessary to support growth, health and life of mammals, and a state of deficiency or excess of this vitamin can constitute a serious threat to health (Machlin, 1991; Geraci, 1981, McDowell, 2000). This fat-soluble vitamin plays a direct role in the maintenance of vision and epithelial tissues, reproduction, bone development and immune system function (Machlin, 1991; McDowell, 2000). In most species, there is a strong homeostatic control of serum vitamin A with levels commonly maintained within a normal range for the species until liver stores are exhausted. For this reason, serum levels of vitamin A must be interpreted with caution, with low levels being useful mainly as an indication of deficiency (McDowell, 2000). The livers of terrestrial mammals can contain up to 90% of the total body vitamin A, with the remainder stored in kidneys, lungs, adrenals, blood and other organs (Machlin, 1991). In certain pinniped species, this distribution differs with the blubber storing a greater proportion of vitamin A than the liver (Schweigert et al., 1987; Mos and Ross, 2002). Zinc deficiency is known to increase vitamin A requirements in domestic animals (McDowell, 2000). There are no published reports of pathological lesions directly attributable to vitamin A excess or deficiency in cetaceans, but one can predict that vitamin A and related compounds are essential but also potentially toxic. Understanding the dynamics of vitamin A moblization and its distribution in tissues is important in light of recent attempts to employ retinol as a biomarker of organochlorine exposure (Rolland, 2000; Simms and Ross, 2000, Borrell et al., 2002, Tornero et al., 2004b).

Organochlorine contaminants (OCs) are ubiquitous in the environment and have been reported in the blubber of marine mammals worldwide (O'Shea, 1999, AMAP, 2002). These compounds are known to bioaccumulate in the lipids of marine mammals and are present in increasing amounts in the Arctic (AMAP, 2002). Previous studies have demonstrated a negative correlation between organochlorine (OC) and retinol concentrations in marine mammal tissues secondary to the binding of OC to transthyretin (Brouwer et al., 1989; de Swart et al., 1994; Simms and Ross, 2000; Jenssen at el., 2003). Monitoring the relationship between these substances is important as OCs possess the potential to adversely affect marine mammal health (de Swart et al., 1994; O'Shea, 1999; AMAP, 2002) and are known to affect metabolism, reproduction, endocrine function and the immune system of marine mammals (Dierauf and Gulland, 2001).

Vitamin E, another fat-soluble vitamin, is essential for integrity and optimal function of reproductive, muscular, circulatory, nervous and immune systems (Hoekstra, 1975; Sheffy and Schultz, 1979; McDowell, 2000). It has complex interactions in the body involving selenium and the sulfur-bearing amino acids cystine and methionine and also functions as an important biological antioxidant. Vitamin E is involved in membrane structure, prostaglandin synthesis, blood clotting, disease resistance and regulation of DNA synthesis. Dietary requirements depend upon many factors, including levels of poly-unsaturated fatty acids (PUFA), selenium, sulfur amino acids and other antioxidants in the diet (McDowell, 2000). Marine mammals are highly dependent upon adequate vitamin E in their diet to protect their body tissues against oxidative stress (Debier 2002). Selenium exerts a sparing effect on vitamin E and delays onset of deficiency signs, while vitamin E serves a similar role in protecting against selenium deficiency (Machlin, 1991; McDowell, 2000).

The bowhead whale (*Balaena mysticetus*) is an endangered mysticete species that lives in Arctic and sub-Arctic waters. Although the Bering-Chukchi-Beaufort Sea (BCBS) Stock was commercially exploited to near extinction in the late 1800's, it is currently growing (George et al., 2004) and is an important subsistence species to residents of northern Alaska, Russia and Canada. The BCBS stock undertakes a yearly migration from the Bering Sea to the Beaufort Sea to reach the highly productive summer feeding grounds located there, providing the opportunity to obtain high quality biological samples from the Inuit subsistence hunt in the spring and fall.

This study measured the levels of vitamins A and E in serum, liver and blubber (at multiple depths) of subsistence-harvested bowhead whales obtained from 1998-2001. We evaluated the relationships between vitamin concentration and gender, season of collection, age class, reproductive status, as well as the associations between vitamin concentrations and percent lipid and concentrations of OC contaminants and selected essential elements.

3.2 Materials and Methods

3.2.1. Field techniques and blood collection

Blubber, liver and blood samples were collected during the Inuit subsistence hunt in Barrow,

Alaska (spring and fall) from 1998-2001. These sample collections were conducted with permission of the Barrow Whaling Captain's Association and the Alaska Eskimo Whaling Commission through the Department of Wildlife Management (North Slope Borough, Alaska) under the purview of a National Oceanic and Atmospheric Administration (NOAA) permit [#932-1489-00 and 932-1489-03 for the Marine Mammals Health and Stranding Response Program (MMHSRP)]. Blood was collected as soon after death as possible (typically within 2-14 hours) from the palatal sinus into untreated red top vacuum tubes (Vacutainer/BD, New Jersey 07417, USA). The blood was allowed to clot and centrifuged for 10 minutes at 3500 g within 4-6 hours of collection. The serum was then removed and frozen immediately at –20°C (Arctic Research Facility, Barrow, Alaska USA) during the remainder of the field season (~1 month) and then at -80°C in the laboratory in a light-proof container until thawed for analysis (University of Alaska Fairbanks, Fairbanks, Alaska). Blubber samples were taken from an area approximately one meter caudal to the blowhole on the dorsal aspect of the whale. Full thickness samples (epidermis to internal muscular layer) were collected. The epidermis was removed at the level of the papillary dermis and the remaining adipose tissue was divided into fifths of equal depth (Fig. 3.1). The first (BD1), third (BD3) and fifth (BD5) section (BD1 being closest to the epidermis and BD5 being closest to the internal muscular layer underlying the blubber) were analyzed. Liver samples were taken from a central portion of the organ that was most immediately available during butchering.

3.2.2. Serum vitamin analysis

Vitamins A and E were extracted and analyzed using a high-performance liquid chromatography (HPLC)/ultraviolet detection method described in Mazzaro et al., 2003, with some modifications. The volumes of bowhead serum and extraction solvent (methanol) were doubled due to the relatively low concentrations of vitamins in this sample matrix. The internal standard (IS), delta-tocopherol, was added to each sample tube and the residue was dissolved in 100 µl propanol. Additionally, a microsorb C18 resolve column (Varian, Inc., Palo Alto, California, 94304 USA) protected with an Upchurch C18 guard/column (Upchurch Scientific, Oak Harbor, Washington, 98277 USA) was used to separate the vitamins, and detection was 325 nm for retinol and 290nm for tocopherols.

3.2.3. Liver samples

Liver vitamins A and E were extracted by adding 1 ml sodium ascorbate and approximately 0.1g liver to 16 x 100 disposable, borosilicate glass test tubes (VWR Scientific). This mixture was then homogenized using a polytron (Brinkman Instruments, Westbury, NY USA). During homogenization, 2 ml ethylenediaminetetraacetic acid (EDTA) was added to the mixture, along with 1 ml potassium hydroxide (KOH) (50% potassium hydroxide- 50grams KOH in 100ml methanol) and 500µl IS (delta-tocopherol). This total mixture was then mixed using a vortex, capped and incubated in water bath for 15 minutes at 70C to saponify the sample. After saponification, the liver mixtures were cooled on ice and 1-2 crystals of butylated hydroxyanisole (BHA) were added to each tube followed by 3 extractions with 2 ml hexane. The mixture was then completely dried under a gentle stream of nitrogen. The final product was reconstituted with 250µl propanol/dicholoroethane (80/20) and analyzed by HPLC. Ten µl of sample was injected onto a Varian C18 column. Methanol/dichloromethane 80/20 was used as the mobile phase at a flow rate of 1.5ml/min. All samples were analyzed in triplicate.

3.2.4. Epidermal/blubber vitamin analysis

Epidermis: approximately 0.25g epidermis, 1 crystal of BHA, 2.5ml KOH (100ml ethanol/16g KOH) and 100 µl of IS (delta-tocopherol) were added to a 20 ml screw top glass test tube.
Blubber: 0.25-0.30 g of blubber, 1 crystal of BHA, 2.5-3.0 ml KOH (100ml Ethanol/16g KOH) were added to a 20 ml screw top glass test tube.

The mixtures were saponified in a water bath at 80°C for 30 minutes and then cooled in cold water for 5 minutes. Distilled water (2.5 – 3.0 ml) was added to each sample mixture and the hexane layer was removed. Two extractions were then performed by adding 2ml hexane. The remaining material was vortexed for 3 minutes and then centrifuged for 5 minutes at 2,500rpm. The mixture was evaporated under a gentle stream of nitrogen and reconstituted with 100µl methanol/dichloromethane (9:1). The sample (50µl) was then injected into HPLC column (5 um long column) with guard column (5um 150x4.6mmx1/4" Varian C18 resolve column), at a flow rate of 1.5 mL/minute using a mobile phase of methanol/water (98/2). Samples were analyzed in triplicate using the following standards: Retinol (325nm), delta-tocopherol (298nm), alpha-tocopherol (292nm). Detector was set at 325nm and 290nm, respectively.

An external standard was used for all samples. Blanks were run alongside the samples to calculate recoveries.

3.2.5. Organochlorine analyses

The OC dataset used in this study was previously reported in Hoekstra et al. (2002). The twenty PCB metabolites and OC congeners occurring in highest mean concentrations in serum, blubber and liver were selected for multivariate analysis (canonical correlation). These organochlorines were detected at low levels in most whales and many are known to have a relatively high affinity for the T4 receptor on the transthyretin (TTR) carrier (Ishihara et al., 2003).

3.2.6. Age determination

Stable isotopes of carbon signature analyses of baleen and aspartic acid racemization of eye lens nuclei were used independently to determine age (George et al., 1999, Rosa et al., 2004, Lubetkin et al., 2004) in the majority of whales (36/58). The remaining whales in this study were designated juvenile, subadult, or adult using a combination of body length, baleen length and gonadal size/development (Table 3.1). Adults were considered to be sexually mature via histologic and morphologic assessment of reproductive tissues.

3.2.7. Essential element analysis

Selenium and zinc were analyzed according to methods detailed in Dehn et al., 2005.

3.2.8. Blubber lipid quantification

Analyses of lipid percentage for each blubber layer (except epidermis) were conducted according to methods described in Krahn et. al (2001) and Ylitalo et al. (2005).

3.2.9. Statistical Analyses

Data are presented as the mean and standard deviation (SD). All data were analyzed by a three-way analysis of variance (GLM, general linear model) using the SAS® system (SAS Institute Inc, Cary, North Carolina 27513, USA) with interaction terms (age class * sex * season). Multivariate relationships were investigated via cannonical correlation, also using SAS. Wilks's lambda was used to test the significance of the first canonical correlation and a likelihood ratio test was used to test the linear relationship between the canonical variables. For the few samples with undetectable levels of OCs (n=16, 4

%), the mean was calculated using a value one-half the minimum detectable level for those undetected values (Gilbert, 1987). A probability of < 0.05 was considered significant.

3.3 Results

Tables 3.2 and 3.3 summarize the retinol and tocopherol results from samples collected in the spring and fall during the four years. The form of vitamin A recovered was retinol [no Vitamin A_2 (didehydroretinol) was detected]. No retinyl esters were found in the serum. The only form of vitamin E recovered was alpha-tocopherol (γ- and δ- tocopherol were not detected). In general, males had significantly higher mean concentrations of hepatic retinol than females [5374.20 μg/g (n=21), 4057.30 μg/g (n=30), respectively, P=0.04]: however, interactions for sex * season and sex * age class were not significant (P=0.88, 0.99, respectively). Liver retinol was significantly higher in the spring-landed versus fall-landed whales [6192.2 μg/g (n=22), 3391.3(n=29) μg/g, respectively, P=<0.0001]. Non-pregnant female and male adults had the highest mean concentration of liver retinol, with the subadults of both sexes, pregnant females and juveniles of both sexes following in decreasing order (these sample sizes did not all for statistical testing of pregnant versus non-pregnant females). The age classes (non-pregnant adults>subadults> juveniles=pregnant females) had significantly different liver retinol levels (Table 3.2). There were no significant differences between age class, sex or seasonal groupings with respect to vitamin E concentrations, with the exception of the intermediate layer of blubber described below. Mean tocopherol concentration in the intermediate blubber layer (BD3) was significantly higher in adults than in subadult or juvenile animals.

Vitamin concentrations by blubber depth are summarized in Table 3.4. Mean retinol concentration (across all age/sex/seasonal groups) was significantly higher in the epidermis (1.65 ± 0.64 μg/g) than in the deeper blubber layers (BD1: 1.60 ± 0.9 μg/g , BD3: 0.69 ± 1.06 μg/g, BD5: 0.50 ± 0.69 μg/g). Tocopherol was significantly higher in epidermis (17.24 ± 5.98 μg/g) and the intermediate layer of blubber (BD3: 15.91 ± 10.30 μg/g) than in the innermost and outermost blubber layers (BD1: 9.60 ± 8.60 μg/g, BD5: 12.19 ± 8.63 μg/g).

No correlation was found between hepatic Zn and serum vitamin A concentrations (R^2=-0.26,

P=0.23). A strong positive correlation was found between liver tocopherol and renal Se concentration (R^2=0.76, P=0.0003). Positive correlations were also noted between hepatic Zn and hepatic retinol (R^2=0.64, P=0.0003) and tocopherol levels (R^2=0.58, P=0.0012). There was a positive correlation found between retinol and tocopherol in both the liver and serum samples (R^2= 0.65, P=<0.0001, R^2= 0.64, P=< 0.0001, respectively). There was no correlation between these two vitamins in the blubber at any depth (BD1: R^2=0.08, P= 0.69, BD3: R^2=-0.05, P=0.83, BD5: R^2=0.07, P=0.74). There was no correlation between blubber percent lipid and the retinol or tocopherol concentrations in the corresponding layers (Table 3.5). There was no correlation found between vitamin A (serum, liver and blubber) and any of the OC metabolites/congeners examined (serum, liver and blubber).

3.4 Discussion

Vitamin A and E concentrations in the bowhead whale varied widely with some variability explained by sex, season, age class and reproductive state. Hepatic retinol had the most significant associations present and these related to sex, season and age group. Serum retinol and tocopherol concentrations were the least variable of the measured values with reported ranges similar to those found in terrestrial mammals (LeBlanc et al., 2004; Yang et al., 1992). Serum retinol is homeostatically regulated with respect to fluctuations in food intake and life history events in many mammalian species (Machlin, 1991; Borrell et al. 2002). The hepatic retinol concentrations listed in Table 3.2 are relatively high in comparison to values reported in domestic mammals, pinnipeds and other cetaceans (Machlin, 1991; McDowell, 2000; Tornero et al., 2004a; Borrell et al, 2002; Borrell et al., 1999; Crissey and Wells, 1985). However, published "reference ranges" must be interpreted with caution due to likely differences among populations and individuals. In addition, inter-laboratory analytical variations make it difficult to adequately compare published values (Ullrey et al., 1995).

Male bowhead whales had significantly higher mean concentrations of liver retinol than females. Similar results have been noted in pinnipeds (Rodahl and Davies, 1949; Schweigert et al., 1987). Lactational loss of retinol may be responsible for this difference (Simms and Ross, 2000). Marine mammals produce milk that is extremely rich in fat. For example, percent fat ranges from a low of 20-25%

in sea otters to 30-60% in pinnipeds and whales (Oftedal, 1984; Debier et al., 1999, 2004). Reproductively mature female marine mammals are known to apportion large amounts of fat and fat-soluble vitamins to offspring via the milk during lactation (Debier et al., 1999, 2002). This may result in a cyclical and possibly cumulative decrease in maternal levels of fat soluble vitamins that is dependent upon life history traits including the length of lactation, calving interval and the number of total lactational periods experienced.

Liver retinol concentration was also significantly higher in whales harvested in spring than in fall. Migration requires a high level of energy expenditure and draws heavily upon body lipid reserves (Burns et al., 1993). Decreased hepatic retinol concentrations in the fall may be related to fat mobilization during the spring/summer migration resulting in a redistribution of blubber and liver associated retinoids (Borrell et al., 1999). Repletion of blubber stores of vitamin A during feeding throughout summer and early fall, prior to the fall harvest is likely. However, the dynamics of the distribution of retinol to organs in cetaceans are largely unknown. Blubber seems to be more stable, with respect to concentration, as there were no seasonal differences in vitamin A and E distribution in this tissue. The liver may take longer than blubber to restore its pre-migration retinol levels as a specific strategy for dealing with a large influx of vitamins during a relatively short period of time. This may be of benefit to these whales, especially with respect to the avoidance of vitamin A toxicity.

Adult bowhead whales have the highest concentrations of hepatic retinol, followed by subadults, pregnant females and juveniles. The effects of age on retinol concentration have been researched in humans and laboratory animals, with a general trend towards an increase in hepatic concentrations with age (van der Loo et al., 2004). The majority of pinniped research supports these findings (Kakela et al., 1997; Southcott et al., 1974; Schweigert et al., 1987; Rodahl and Davies, 1949) and indicates a cumulative increase in liver retinol concentrations with feeding over a lifetime (Southcott et al, 1974, Schweigert et al., 1987, Borrell et al., 1999). This may be especially important to bowhead whales, due to their extreme longevity (George et al., 1999; Rosa et al., 2004). Additionally, there may be decrease in circulatory clearance of retinoids with increasing age. Higher tissue concentrations of retinol might also be a defensive adaptation to protect against oxidative tissue damage that becomes more prevalent with age (van der Loo et al., 2004). Juvenile bowhead whales had the lowest levels of hepatic retinol. This age group receives

appreciable amounts of vitamin A via milk during the nursing period (Debier et al., 2002): however, the transfer of retinol across the placenta is minimal and mammalian neonates are born with very low liver stores of vitamin A (McDowell, 2000). Time is needed to build up a cumulative body store of vitamin A via nursing and foraging following weaning.

Liver oils of fish and marine mammals contain some of the richest sources of vitamin A known (McDowell, 2000). Much of the published research investigating vitamin A concentrations in marine mammals has focused on pinnipeds (Schweigert et al., 1987, Ball et al., 1992, Mazzaro et al., 2003) with published liver retinol concentrations up to 100 times the levels found in terrestrial mammals (McDowell, 2000; LeBlanc et al., 2004; Yang et al., 1992). The diet of the bowhead whale is composed mainly of euphausids, amphipods, copepods, and mysid shrimp (Burns et al., 1993, Richardson and Thomson, 2002). These prey items contain relatively high levels of vitamins A and E (Krinsky, 1965, Kon and Thompson, 1949), which is important, as retinol cannot be produced endogenously in most mammals and must be provided in the diet. Vitamin A is stored primarily in the liver in the majority of mammals, with pinnipeds appearing to be an exception (McDowell, 2000, Schweigert et al., 1987; Mos and Ross, 2002). In seals and sea lions, blubber is of greater significance than liver as a storage depot for vitamin A with between 40 and 60 percent of total body stores being found in this tissue in grey (*Halichoerus grypus*) and harbor seals (*Phoca vitulina*) (Schweigert et al., 1987, Mos and Ross, 2002). Cetaceans also store vitamin A in their blubber but not at the comparatively high concentrations found in pinnipeds (Tornero et al., 2004a, 2004b). This is consistent with our findings in the bowhead whale. However, because blubber comprises 50-60% of total body mass in the bowhead (J.C. George, personal communication) this is a more significant storage site in this species than in many other cetaceans. Previous research has shown a correlation between retinol and percent lipid in blubber of common dolphins (*Tursiops truncatus*) (Tornero et al., 2004b). However, this relationship was not found in hooded seals (*Cystophora cristata*), harbor porpoises (*Phocoena phocoena*) or harbor seals (*Phoca vitulina*) (Rodahl and Davies, 1949, Borell et al., 1999, Mos and Ross, 2002). Similar to these findings, no correlations were found between retinol or alpha-tocopherol and blubber lipid percentage in the bowhead whale (Table 3.5).

In the present study, retinol and tocopherol concentrations were found to be significantly higher in the epidermis than in the deeper blubber layers. This is similar to findings in several other mammalian species, which tend to concentrate these substances in the epidermis (Vahlquist et al., 1987). The bowhead whale has among the thickest epidermal tissue of all mammals (Haldiman et al., 1985), making this tissue a sizeable depot for these vitamins. Both substances are likely to be important for growth, maintenance of skin, and wound healing (Machlin, 1991; McDowell, 2000). The concentration of retinol in the outermost blubber layer (BD1) was significantly higher in adults than in subadult and juvenile bowhead whales. This outermost region of blubber is thought to serve as a potential long-term storage or highly lipid stable region (Mau, 2004; Ackman et al, 1975) and may be higher in retinol secondary to age-dependent accumulation. Tocopherol was significantly higher in the intermediate layer of blubber (BD3) than in the innermost and outermost blubber layers. The intermediate layer of blubber has been shown to contain the highest percentage of lipid in the bowhead whales studied (Mau, 2004) though there was no correlation noted between tocopherol and percent lipid of the corresponding blubber layers. The tocopherol concentration in blubber layer three (BD3) was found to be significantly higher in adults than those subadult and juvenile bowhead whales. This may be an age-related finding, as well, as tocopherol has been found to accumulate with age in the blubber of marine mammals (Kakela et al., 1997; Schweigert et al., 1990). More information is needed on the mobilization of blubber lipids in order to fully characterize these processes.

In pinnipeds, hepatic vitamin E is thought to increase transiently after ingestion of tocopherol –rich food. Subsequently, hepatic levels drop and blubber levels rise, with the blubber acting as a long-term storage depot for the vitamin (Engelhardt et al., 1975; Käkelä et al., 1997; Schweigert et al., 2002). We found that the blubber of bowhead whales contained a much lower concentrations of vitamin E than those reported in seal blubber (Crissey and Wells, 1999; Scweigert et al., 1990; Engelhardt et al., 1977; Schweigert et al., 2002). In contrast, these whales had up to ten times the hepatic concentration of tocopherol reported in seals both during the spring (fasting/transient feeding) and fall (feeding) periods. Available literature contains no vitamin E data for cetacean tissues except serum.

Vitamin E and selenium interact in tissues and are thought to be mutually protective of each other with respect to deficiency. Notable correlations were found for bowhead liver tocopherol levels and renal

selenium levels, hepatic Zn and hepatic retinol and hepatic Zn and hepatic tocopherol levels in the current study. No correlation (positive or negative) was found between hepatic Zn and serum vitamin A concentrations. Reasons for the above correlations are unknown though one may speculate that these substances may occur in specific ratios in prey and are distributed in similar ways throughout the body of the predator (bowhead whale). Alternativley, an age-related accumulation of these elements may occur.

Levels of hepatic tocopherol were high in bowhead whales compared to those of terrestrial mammals. This may reflect the fact that many of these marine mammals are consuming diets high in PUFAs. The most important determinant of vitamin E requirements is the dietary concentration of unsaturated fatty acids as PUFAs are highly susceptible to auto-oxidation (Nacka, 2001). Animals ingesting high levels of PUFAs require high concentrations of vitamin E to protect tissue lipids from free radical attack (Debier 2002, Lammi-Keefe and Jensen, 1984; Machlin, 2000). Tocopherol levels in the bowhead whale were found to be stable over sex, seasonal, age and reproductive groups.

Bowhead whales feed at a low trophic level and are known to have OC concentrations that are an order of magnitude lower than closely related mysticetes from the Atlantic waters (Hoekstra et al, 2002). However, they are also long-lived, have a large amount of lipid incorporated in blubber and are thought to have low metabolic rates (J.C. George, personal communication), all of which may affect their accumulation, metabolism and clearance of OCs. In our analyses, retinol concentration in the serum, liver and blubber did not correlate with any of the 20 OCs or PCB congeners measured in serum and blubber. This lack of correlation is likely to be related to the low levels of OCs found in this sample. An alternative explanation is that retinol levels in the bowhead whale are not affected by organochlorines; this is unlikely since it is contrary to most of the marine mammal literature. Further investigation into the significance of the transthyretin (TTR) carrier specificity for retinol in marine mammals is needed. Longitudinal, long-term studies monitoring OC concentrations in tissues (through the subsistence hunt and skin biopsy collection), with additional work investigating the status of retinol transport proteins and distribution patterns will help identify these relationships and ultimately the usefulness of retinol as a biomarker in this species. Adverse health effects may occur in marine mammals secondary to abnormalities in vitamin A and E status. Understanding of the dynamics, tissue distribution and baseline levels of vitamin A and E in mysticetes is

critical to interpretation of these changes. The relationship of these findings to organochlorine concentrations helps to assess their utility as biomarkers of exposure to OCs and indicators of health in cetaceans.

3.5. Acknowledgements

We express our appreciation to the Department of Wildlife Management, North Slope Borough (Barrow,v Alaska), the Cooperative Institute for Arctic Research (CIFAR), the Alaska IDeA Networks for Biomedical Research Excellence (INBRE) and NOAA for generous funding and logistical support. We also thank the subsistence whalers in Barrow and Kaktovik (Alaska USA) and the Alaska Eskimo Whaling Commission (AEWC) for graciously allowing us to access these samples. Individual thanks to Victoria Woshner, Lara Dehn, Mike Castellini, Douglas DeMaster, Cyd Hans, Craig George, Benny Akootchook, Gladys Yanagida and Chris Terzi for their help with the manuscript, tissue collection and analytical support. Bowhead whale samples were collected under the authority of permit #932-1489-00 and #932-1489-03 for the MMHSRP program (Dr. Teri Rowles who was instrumental in project development). Support for this project came from CIFAR and Alaska INBRE grant number 2 P20 RR016466 from the National Center for Research Resources, National Institutes of Health, Dept. of Health and Human Services.

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Table 3.1.
Bowhead whale age group definitions as determined via aspartic acid racemization , carbon baleen isotope ($\delta^{13}C$)measurement and histological analyses.

Juvenile:	1-3 year old whales that experience an accelerated period of growth
Subadult:	3 years of age to ~22 years of age in males and ~25 years of age in females (sexually immature)
Adult:	greater than ~22 years of age in males and ~25 years of age in females (sexually mature)

(George et al., 2001, Lubetkin et al. 2004, Rosa et al., 2004)

Table 3.2. Retinol concentrations in the serum, liver, epidermis and blubber of the bowhead whale. Blubber samples include BD1 (outermost depth), BD3 (middle depth) and BD5 (innermost depth). Mean ± standard deviation followed by sample size in class in parentheses below the value.

	serum (μg/ml)	liver* (μg/g)	epidermis (μg/g)	BD-1 (μg/g)	BD-3 (μg/g)	BD-5 (μg/g)
Adult						
Female						
Non-pregnant	0.10 (n=1)	7568.67±3801.36 (n=3)	-	1.87 (n=1)	0.06 (n=1)	0.09 (n=1)
Pregnant	0.09±0.02 (n=4)	4385.50±3105.92 (n=4)	2.93 (n=1)	-	-	-
Male	0.09±0.04 (n=6)	7261.33±3134.62 (n=6)	1.47±0.81 (n=2)	3.09±0.49 (n=2)	1.79±2.31 (n=2)	0.99±1.15 (n=2)
Subadult						
Female	0.10±0.03 (n=6)	5349.80±4151.83 (n=5)	1.96±0.40 (n=2)	1.98±0.54 (n=4)	1.51±1.67 (n=4)	1.12±0.96 (n=4)
Male	0.10±0.03 (N=5)	5132.40±4971.06 (n=5)	1.67±1.17 (n=2)	0.64±0.92 (n=3)	0.16±0.06 (n=3)	0.09±0.13 (n=2)
Juvenile						
Female	0.09±0.02 (n=14)	3040.06±1830.55 (n=18)	1.50±0.59 (n=10)	1.44±0.74 (n=9)	0.18±0.11 (n=10)	0.17±0.12 (n=10)
Male	0.09±0.03 (n=17)	4362.80±2747.15 (n=10)	2.89±2.89 (n=5)	1.49±0.23 (n=3)	1.07±0.88 (n=3)	1.09±1.21 (n=2)

* denotes a significant difference for this variable between non-pregnant adult, subadult and juvenile age classes with adults>subadults>juveniles=pregnant females

Table 3.3. Alpha–tocopherol concentrations in the serum, liver, epidermis and blubber of the bowhead whale. Blubber samples include BD1 (outermost depth), BD3 (middle depth) and BD5 (innermost depth). Mean ± standard deviation followed by sample size in class in parentheses below the value.

	serum (μg/ml)	liver (μg/g)	epidermis (μg/g)	BD-1 (μg/g)	BD-3* (μg/g)	BD-5 (μg/g)
Adult						
Female						
Non-pregnant	20.26 (n=1)	678.67±275.62 (n=3)	-	8.17 (n=1)	12.42 (n=1)	15.55 (n=1)
Pregnant	25.37±14.55 (n=4)	587.25±715.89 (n=4)	19.09 (n=1)	-	-	-
Male	21.21±14.65 (n=6)	954.83±690.62 (n=6)	20.84±10.54 (n=2)	1.75±2.47 (n=2)	34.61±14.97 (n=2)	15.58±5.20 (n=2)
Subadult						
Female	19.63±14.39 (n=6)	920.40±444.20 (n=5)	15.99±0.11 (n=2)	8.05±7.48 (n=4)	10.15±7.53 (n=4)	6.41±4.32 (n=4)
Male	19.49±12.16 (n=5)	546.00±351.71 (n=5)	15.67±6.60 (n=2)	11.77±4.95 (n=3)	14.19±7.48 (n=3)	21.91±15.70 (n=2)
Juvenile						
Female	17.97±10.38 (n=14)	396.16±335.98 (n=18)	16.15±4.67 (n=10)	11.71± (n=10, 11.43)	16.08± (n=10, 10.24)	10.86± (n=10, 7.56)
Male	16.50±9.81 (n= 17)	708.00±512.90 (n=10)	19.12±10.01 (n=5)	8.21±4.06 (n=3)	13.46±2.71 (n=3)	15.66±18.02 (n=2)

* Adults had a significantly higher alpha-tocopherol concentrations than subadults or juveniles in the BD3 blubber layer

Table 3.4. Distribution of retinol and ∝–tocopherol in blubber layers of the bowhead whale. Blubber samples include BD1 (outermost depth), BD3 (middle depth) and BD5 (innermost depth). Mean ± standard deviation followed by sample size in class in parentheses below the value.

	Epidermis*, ** (μg/g)	BD-1 (μg/g)	BD-3** (μg/g)	BD-5 (μg/g)
Adult				
Retinol	1.47±0.81 (n=2)	2.68±0.79 (n=3)	1.21±1.91 (n=3)	0.69±0.96 (n=3)
∝–tocopherol	20.84±10.54 (n=2)	3.89±4.10 (n=3)	27.21±16.62 (n=3)	15.57±3.68 (n=3)
Subadult				
Retinol	1.82±0.73 (n=4)	1.40±0.97 (n=7)	0.93±1.39 (n=7)	0.78±0.93 (n=6)
∝–tocopherol	15.83±3.82 (n=4)	9.64±6.33 (n=7)	11.88±7.19 (n=7)	11.58±11.16 (n=6)
Juvenile				
Retinol	1.96±1.75 (n=15)	1.45±0.65 (n=13)	0.38±0.54 (n=13)	0.32±0.52 (n=12)
∝–tocopherol	16.99±6.34 (n=14)	10.90±10.16 (n=13)	15.47±9.01 (n=13)	11.66±8.93 (n=12)

*Mean retinol concentration (across all age/sex/seasonal groups) was significantly higher in the epidermis than in the deeper blubber layers.
**Tocopherol was significantly higher in epidermis (17.24 ± 5.98 μg/g) and the intermediate layer of blubber (BD3: 15.91 ± 10.30 μg/g) than in the innermost and outermost blubber layers

Table 3.5. Spearman rank correlation values (noted as R) found between blubber retinol, blubber tocopherol and blubber % lipid in the bowhead whale (significance $P < 0.05$.)

		blubber retinol depth 1	blubber tocopherol depth 1	blubber retinol depth 3	blubber tocopherol depth 3	blubber retinol depth 5	blubber tocopherol depth 5
BD1	R	0.178	0.01	0.22	-0.11	-0.02	-0.22
Lipid %	*P*	0.43	0.97	0.34	0.61	0.94	0.40
	n	22	20	21	23	15	16
BD3	R	-0.15	0.10	0.22	-0.22	-0.14	-0.17
Lipid%	*P*	0.49	0.67	0.34	0.30	0.61	0.52
	n	22	20	21	23	15	16
BD5	R	0.03	0.09	0.25	-0.18	-020	-0.29
Lipid %	*P*	0.88	0.72	0.28	0.41	0.46	0.28
	n	22	20	21	23	15	16

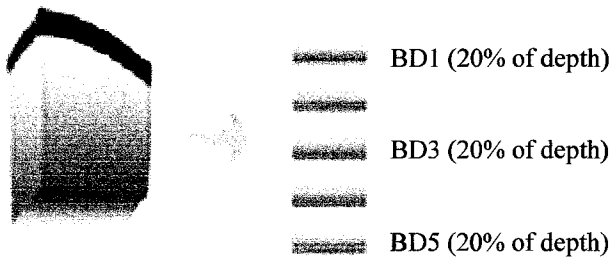

Figure 3.1.

Blubber core sampling scheme in the bowhead whale. Each core had the epidermis removed (this was analyzed separately) and the remaining blubber was measured and divided into 5 equal sections, each representing 20% of the core.

3.7 Appendix

List of organochlorine congeners and PCB metabolites (liver, blubber and serum) analyzed by cannonical correlation analyses in the bowhead whale liver, serum and blubber.

op-DDD
ppDDE
a-HCH
HCB
b-HCH
Oxychlordane
cis-Heptachlorepoxide
Dieldrin
pp-DDD
Trans-Nonachlor
g-Chlordane
op-DDT
1-4-DCB
c95&c66
g-HCH(Lindane)
c52c49
pp-DDT
op-DDE
c101
Methoxyclor

Chapter Four

Serum haptoglobin detection in the bowhead whale (*Balaena mysticetus*)[3]

4.0 Abstract

As a component of a larger health assessment study, we used a haptoglobin-hemoglobin binding assay to analyze serum from 51 bowhead whales (*Balaena mysticetus*) for the presence of haptoglobin. Haptoglobins are serum glycoproteins that are produced during the acute phase reaction and are increased when there is extensive tissue damage or necrosis. Haptoglobins may be absent or present at low levels in the serum of healthy individuals, depending upon species.

Whales in this sample were landed over a three-year period (1998-2000) in Northern Alaska by native subsistence hunters. Haptoglobin was identified in the serum of 3/51whales analyzed. This suggests that an active inflammatory process may have been present at the time of capture in these 3 individuals. These results are evaluated with respect to necropsy and histopathology data and morphometric measurements. The utility of serum haptoglobin measurements in large cetaceans requires further investigation but this relatively simple, inexpensive assay may be a beneficial addition to cetacean population health assessment.

Keywords: acute phase proteins, *Balaena mysticetus*, bowhead whale, cetacean, haptoglobin, inflammation, marine mammal

[3] Rosa, C., Blake, J.E. Serum haptoglobin detection in bowhead whale (*Balaena mysticetus*) serum Prepared as a short communication for the Journal of Wildlife Diseases

4.1 Introduction

Acute phase proteins function in the regulation of immune responses, mediation and inhibition of inflammatory processes. They also act as transport proteins for products generated during the inflammatory process and play an active role in tissue repair and remodeling (Wassell, 2000). Elevated serum concentrations of certain acute phase proteins are of diagnostic and prognostic value (Alsemgeest and Gruys, 1990; Asai et al., 1999; Solter et al., 1991) and allow inflammatory processes to be distinguished from functional disturbances with similar clinical appearances. Under normal circumstances, an acute phase response is not observed with functional disturbances that are not a result of an inflammatory process, thereby allowing the differentiation between failure of function and organic disease (Zento-Savin et al., 1997; Solter et al., 1991).

Haptoglobin is one of the acute phase proteins produced during the acute phase reaction. Its synthesis is induced by various cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6) and ciliary neurotrophic factor. Serum haptoglobin concentration rises in response to tissue damage or necrosis in most species studied (Alsemgeest and Gruys, 1990; Asai et al., 1999; Solter et al., 1991). Haptoglobins have been measured in several domestic and laboratory species (Mominoki et al., 1995; Katnik et al., 1998; Solter et al., 1991). Several wild mammal species have been investigated as well. Levels of haptoglobin were increased in the blood of river otters (*Lutra canadensis*) living in oiled areas of Prince William Sound, Alaska after the Exxon Valdez Oil Spill (Duffy et al., 1994a,b). Haptoglobin levels were significantly higher in Steller sea lions (*Eumetopias jubatus*) and harbor seals (*Phoca vitulina*) that were sampled in areas of population decline relative to those from stable populations (Zento-Savin et al., 1997). In cetaceans, haptoglobin has been identified via polyacrylamide gel electrophoresis in sei (*Balaenoptera borealis*), sperm (*Physeter macrocephalus*), humpback (*Megaptera noveangiae*) and fin whales (*Balaenoptera musculus*) (Travis et al., 1971). Our objectives were to determine if haptoglobin could be detected in the bowhead whale (*Balaena mysticetus*) using a hemoglobin-binding assay and to establish if the presence of haptoglobin corresponds with evidence of illness or inflammation as assessed via histology, toxicology, serum chemistry and other health assessment parameters. With these data, we then explored the clinical relevance in measuring serum haptoglobin in cetacean species.

4.2 Materials and Methods

Blood samples were collected during the 1998-2000 spring and fall Inuit subsistence harvest in Barrow and Kaktovik, Alaska. These sample collections were conducted with the permission of the Barrow and Kaktovik Whaling Captains Associations and the Alaska Eskimo Whaling Commission through the Department of Wildlife Management (North Slope Borough, Alaska) under the purview of a National Oceanographic and Atmospheric Association (NOAA) permit issued to Dr. Teri Rowles (permit #932-1489-00 and #932-1489-03 for the MMHSRP program). Blood was collected approximately 2-14 hours post-mortem from the palatal sinus of 51 bowhead whales into untreated evacuated red top tubes (Vacutainer/BD, New Jersey USA). The blood was allowed to clot and was centrifuged within 4-6 hours of collection. The serum was transferred by pipette to a 5ml plastic culture tube and frozen and archived at –20C (initial 2-4 weeks) and –80C (remainder of time) until thawed for haptoglobin analyses. All analyses were conducted in 2001.

Serum haptoglobin was assayed using a commercially available test kit (Helena Laboratories Test for Haptoglobins, Beaumont, Texas USA). For this test we used reindeer (*Rangifer tarandus*) hemoglobin (Hb) in the binding assay. Hemoglobin was prepared for addition to each serum sample as follows: Blood (5 ml) was collected from a normal healthy reindeer and placed in a lavender topped vacuated blood collection tube containing EDTA (Vacutainer/BD, New Jersey USA). The tube was centrifuged for 10 minutes at 3500 g and plasma decanted and discarded. The remaining red blood cells were washed 3-4 times with saline (0.85% NaCl) using a 1:10 ratio of blood to saline; spinning and collecting supernatant after each wash. After the final wash, distilled water was added in a ratio of 2 parts water:1 part blood cells. The tube was then shaken and frozen to hemolyze the cells. The solution was thawed at room temperature, centrifuged and the supernatant was decanted (the pellet that formed at the bottom of the tube was discarded). The concentration of the Hb in the supernatant was measured (approximately 10g Hb/100 ml) using a hemoglobinometer. Hemoglobin (2µl) was added to the serum sample (38µl) to bind to any haptoglobin present in the sample. The haptoglobin-Hb complex was separated from remaining Hb on a polyacrilamide gel using electrophoresis (gel run for 45 minutes at 105-110 V). The plate was stained immediately following electrophoresis and allowed to dry overnight. A negative and positive control

sample (reindeer) was included on each gel. Results were considered positive if a separation was visualized from the control sample included on each gel.

4.3 Results

Serum haptoglobin levels were measurable in bowhead whales using the hemoglobin-haptoglobin binding assay. The band representing the hemoglobin-haptoglobin complex separated well from the hemoglobin band following electrophoresis (Figure 4.1). Serum samples were analyzed from 51 bowhead whales with three reactors detected.

4.4 Discussion

Health assessment data in marine mammals can be difficult to interpret, as information is commonly available from sick or stranded individuals with little baseline data existing or obtainable from healthy conspecifics. The bowhead whale subsistence hunt provides a unique opportunity to collect and evaluate blood, tissues and morphometric data from healthy individuals in order to evaluate the utility of serum haptoglobin as a diagnostic tool. Three of the fifty-one whales (~6%) sampled reacted positively for haptoglobins. It was our hope to compare histological data from these reactors with haptoglobin-negative animals in order to link actual pathological processes taking place in these whales. However, this objective was difficult to realize as the three reactors had a sampling regime limited to morphometric data collection, gross necropsy and blood samples. None of the haptoglobin-negative whales were found to have gross pathological lesions that could be consistent with haptoglobin stimulation. Many of the non-reactors had histology available (32/48), and no evidence of inflammatory/infectious processes was noted histologically in these animals (Rosa, 2005 unpublished data).

Despite the lack of histology in the haptoglobin-positive animals, we compared serum chemistry, morphological data and gross necropsy findings. A subset (n=10) of the haptoglobin-negative whales was found to be within a normal range of serum biochemical values. Two of the three haptoglobin positive animals were found to have abnormal serum chemistry values (creatinine, aspartate aminotransferase, creatine phosphokinase), as compared to normal bowhead chemistry ranges (Heidel et al., 1996). One of the three haptoglobin-positive whales (00B2) had numerous scars and ectoparasites upon capture. However, lack of histological data on the lesions in the positive whales makes interpretation of these data

difficult. Another haptoglobin-positive whale (00KK3) was morphometrically smaller than other whales in its length class. Lower weight/size or poor growth may relate to poor nutrition or ill health. The third whale (00B14) that was haptoglobin positive had thyroid hormone concentrations considerably lower than the mean concentrations sampled for his age group (00B14: total thyroxine=49, free thyroxine=16, age class sample means were 91 and 25 respectively) (Rosa, unpublished data) which could also be indicative of health problems. These are all health parameters that could be related to illness, but it is important to note that no clear connection can be made between the positive reactors and systemic illness without additional (histological, biochemical) evidence. More research is needed, but preliminary results do not rule out haptoglobin analysis as a potentially quick, easy and inexpensive means of providing additional information on the health of marine mammal populations.

4.5 Acknowledgements

We express our appreciation to the Department of Wildlife Management, North Slope Borough (Barrow, Alaska), the Cooperative Institute for Arctic Research (CIFAR), UAF INBRE (Idea Networks for Biomedical Research Excellence) and NOAA for generous funding and logistical support. We thank the Inupiaq subsistence whalers in Barrow and Kaktovik and the Alaska Eskimo Whaling Commission (AEWC) for graciously allowing us to access these samples. Individual thanks to Gay Sheffield, Victoria Woshner, Lara Dehn, Mike Castellini, Doug DeMaster, Todd O'Hara, Cyd Hans, Craig George, Benny Akootchook and Chris Terzi, for their help with the manuscript, tissue collection and analytical support. Bowhead whale samples were collected under the authority of permit #932-1489-00 and #932-1489-03 for the MMHSRP program (Dr. Teri Rowles). Support for this project came from CIFAR and Alaska INBRE grant number 2 P20 RR016466 from the National Center for Research Resources, National Institutes of Health, Dept. of Health and Human Services.

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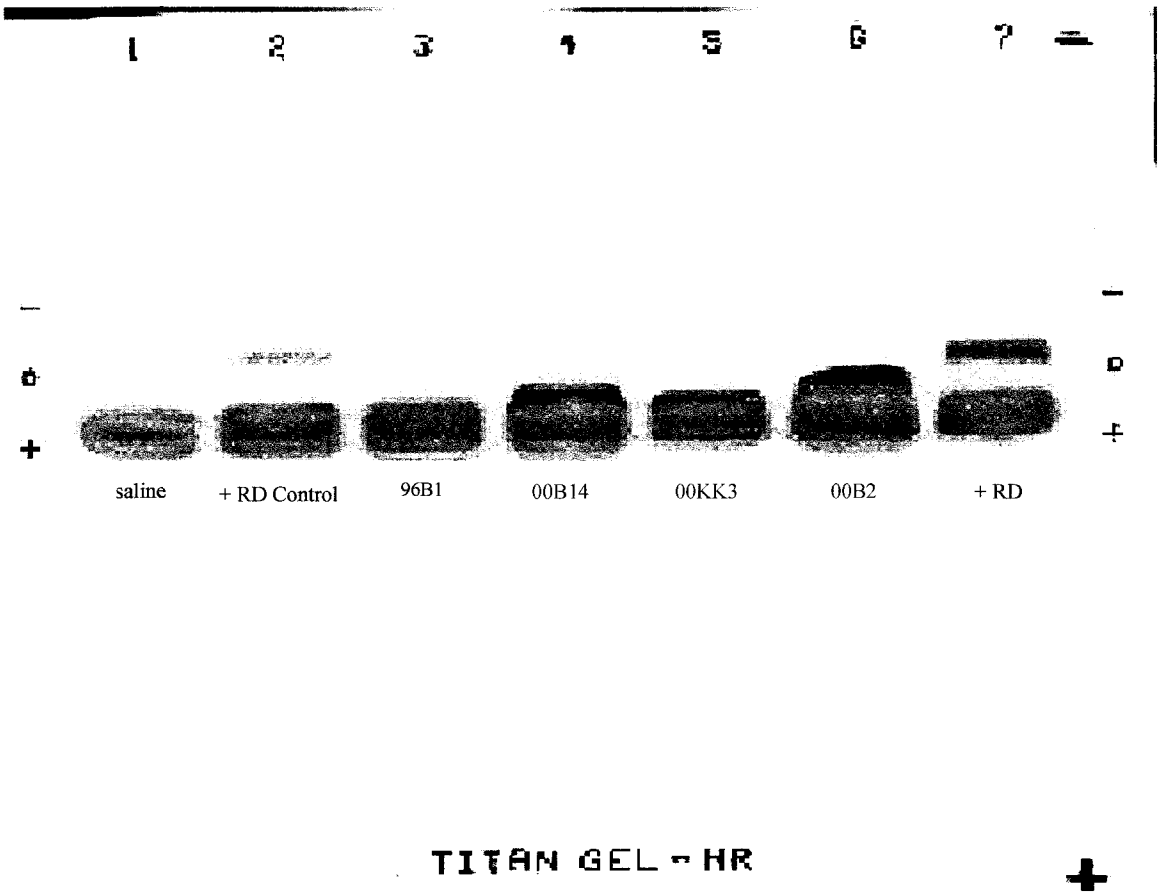

Figure 4.1.
Haptoglobin gel with three positive bowhead whale reactors present (00B14, 00KK3 and 00B2). Whale 96B1 is a negative reactor and the positive reindeer control, saline control and another positive reindeer reactor are included for comparison and quality control purposes.

Chapter Five

Heavy metal and mineral concentrations and their relationship to histopathological findings in the bowhead whale (*Balaena mysticetus*)[4]

5.0 Abstract

The bowhead whale (*Balaena mysticetus*) is an endangered species of great cultural importance and subsistence value to the Inuit of Northern Alaska. This species occupies subarctic and arctic regions presently undergoing significant ecological change and hydrocarbon development. Thus, understanding the health status of the Bering-Chukchi-Beaufort Sea (BCBS) stock of bowhead whales is of great significance. In this study we evaluated the concentrations of six essential and non-essential elements (Zn, tHg, Ag, Se, Cu and Cd) in liver and kidney collected from the Inuit native subsistence hunt in Barrow, Wainwright and Kaktovik, Alaska between 1983 and 2001. Reference ranges of these elements (including previously reported data from 1983-1997) were developed for this species as part of a health assessment effort, and interpreted relative to estimated age (years) to evaluate trends over time with increased statistical power. Interactions between element concentrations and age, sex and season of collection were assessed. Age was found to be the most highly significant factor related to element accumulation (tHg, Cd, Se, Zn). Sex and season of collection were not found to affect the concentrations of these elements, with the exception of renal Se levels, which were significantly higher in the fall.

Histological evaluations of tissues from whales collected between 1998-2001 were performed. Associations between concentrations of Cd in kidney and liver and scored histopathological changes were evaluated. Liver Cd concentration was strongly associated with the degree of lung fibromuscular hyperplasia (P= 0.001), moderately associated with the degree of renal fibrosis (P= 0.03) and were not associated with the degree of liver fibrosis observed (P= 0.06). Renal Cd concentration influenced the degree of lung fibromuscular hyperplasia and renal fibrosis (P=0.01, 0.01, respectively), but did not affect the degree of liver fibrosis (P= 0.14). A significant age effect was found for both pulmonary fibromuscular hyperplasia

[4] Rosa, C., Blake, J.E., Bratton, G.R., Dehn, L-A., Gray, M.J., O'Hara, T.M. Heavy metal and mineral concentrations and their relationship to histopathological findings in the bowhead whale (*Balaena mysticetus*). Prepared for Science of the Total Environment

and renal fibrosis, as well, making age a confounding or causative factor. Incorporating age (in years) and the addition of histological indices help clarify the relationships between elements and the influence of life history parameters on concentrations of these elements and potential impacts on health.

Key words: *Balaena mysticetus*, bowhead whale, cadmium, histology, marine mammals, toxicology.

5.1 Introduction

The toxic effects of metals and their transport through the arctic environment and food chain have been the subjects of recent studies (Mackey *et al.,* 1996; Wagemann *et al.,* 1998; Skaare *et al.,* 1996; Woshner *et al.,* 2001; Dehn *et al.,* 2005a, b). Anthropogenic contamination of air, water, soil and food impact the fauna of the Arctic and subsistence consumers of these animals (Muir *et al.,* 1992; Stirling *et al.,* 1999; Bard, 1999; Dehn *et al.,* 2005b). Heavy metals, including toxic metals, and minerals occur naturally in the environment and many are essential for life (Goyer, 1996). However, certain toxic metals appear to be increasing in the environment (Bard, 1999) and elements that are critical for life at a given concentration may lead to loss of function or death when they occur in greater concentrations (Goyer *et al.,* 1996).

The toxic effect manifested in an organ (and in the organism in general) is a function of exposure as well as time, as many toxicants bioaccumulate. Thus, age is a critical factor in toxicology research. Recent advances in aspartic acid racemization and ^{13}C isotope technology have allowed the development and optimization of an aging protocol in large cetaceans (George *et al.,* 1999; Lubetkin *et al.,* 2004; Rosa *et al.,* 2004). These methods help to minimize the variability associated with using body length as a proxy for age in long-lived cetacean species.

Bowhead whales are mysticetes that feed on a low trophic level (i.e., zooplankton) which affects the concentration of heavy metals and minerals found in their organs (Bratton *et al.,* 1993; Woshner *et al.,* 2001; Krone *et al.,* 1999). However, when the bioaccumulation and biomagnification factors are considered with respect to Cd, bowhead whale tissues have higher Cd concentrations than would be expected as compared to other terrestrial and marine species from higher trophic levels (Bratton *et al.,* 1993; Woshner *et al.,* 2001; Dehn *et al.,* 2005a). Most other elements have been found in low concentrations that are of little concern; however, elements may exert antagonistic or synergistic influences on the accumulation and effects of each other (Ikemoto *et al.,* 2004; Goyer, 1996). Many metals/minerals are known to interact with other elements and proteins: including Se and Hg, and Cu, tHg , Cd and metallothionein (MTH) (Wagemann *et al.,* 1984; Woshner *et al.,* 2001; Ikemoto *et al.,* 2004). These interactions are important to explore, as they may determine the bioavailability of the toxicant or the effect that is seen in a particular species. Marine mammals employ MTH as a physiologic adaptation for dealing with toxicants.

Metallothioneins are proteins that serve metal binding and transport functions and are induced in response to a variety of environmental interactions, including heavy metal, radiation and oxidizing chemical exposure (Teigen *et al.,* 1999). An important role of MTH is in the detoxification and pathogenesis of Cd toxicity (Das et al., 2000). Cd bound to metallothionein is thought to be non-toxic. However, when high concentrations of Cd are reached, MTH's protective effects are overwhelmed and the unbound levels become potentially toxic. This toxicity may manifest itself in many clinical forms, including pulmonary and renal disease (Goyer, 1996; Aughey *et al.,* 1984; Beiglbock *et al.,* 2002; Damek-Poprawa and Sawicka-Kapusta, 2003), as Cd has been found to affect renal proximal tubule function in terrestrial mammals (Goyer, 1996) and has been related to pulmonary and renal fibrosis in laboratory studies of rodents (Goyer, 1996; Aughey *et al.,* 1984; Beiglbock *et al.,* 2002; Damek-Poprawa and Sawicka-Kapusta, 2003).

Although concentrations of heavy metals and minerals in arctic marine mammals have been frequently reported (Braune *et al.,* 1991; Becker *et al.,* 1995b; Dietz *et al.,* 1999a and b; Woshner *et al.,* 2001), a connection between toxicant levels and changes at the organismal level has rarely been made. The relationship between Cd concentration and renal pathology has been examined in ringed seals (*Phoca larga*) (Sonne-Hansen *et al.,* 2002), and organohalogen contaminants and hepatic pathology have been compared in polar bears (*Ursus maritimus*) (Kirkegaard *et al.,* 2005). Otherwise, there has been little research investigating elemental concentrations and associated adverse health effects for individuals and to populations of marine mammals. Severe fibrosis and fibromuscular hyperplasia have been observed in renal and pulmonary tissues in the bowhead whale (Woshner, V. 2000; Rosa, unpublished data). Hepatic lipidosis, pigment accumulation and splenic extramedullary hematopoiesis have also been noted (Woshner, V. 2000; Rosa, unpublished data). The significance of these findings was not interpreted with respect to toxicant concentrations prior to this investigation.

The bowhead whale is a large, endangered mysticete species that occupies arctic and subarctic waters. Bowhead whale blubber is extensively developed, with maximum thickness reported between 43 cm and 50 cm (Lowry, 1993), and the species is known for its longevity, reaching ages upwards of 150 years (George *et al.,* 1999; Rosa *et al.,* 2004). These factors, among others, make them a species of interest with respect to their exposure, metabolism and excretion of toxicants. This study determined the

concentration of 6 essential and non-essential elements (Zn, tHg, Ag , Se, Cu and Cd) in kidney and liver of bowhead whales collected during the Inuit subsistence hunt in Northern Alaska over a 18 year period. Our primary objectives were to investigate the influence of age, sex, and season of harvest on the bioaccumulation of these elements and interpret histological features observed in lung, kidney and liver with respect to these trace element concentrations.

5.2 Materials and Methods

5.2.1 Sample collection

Tissues for chemical analysis were collected during the spring and fall Inuit subsistence hunt in Barrow (71.22°N, 156.00°W) (n=129), Wainwright (70.55°N, 159.95°W) (n=7) and Kaktovik (70.10°N, 43.23°W) (n=9), Alaska between 1983 and 2001 (Fig. 5.1). Tissues for histological analysis were collected during the 1998-2001 spring and fall Inuit subsistence hunt in Barrow and Kaktovik, Alaska. These sample collections were conducted with permission of the Barrow and Kaktovik Whaling Captain's Associations and the Alaska Eskimo Whaling Commission through the Department of Wildlife Management (North Slope Borough, Alaska) under the purview of a National Oceanic and Atmospheric Administration (NOAA) permit [#932-1489-00 and 932-1489-03 for the Marine Mammal Health and Stranding Response Program (MMHSRP)]. Tissue samples collected for histological analysis were placed in 10% neutral buffered formalin within approximately 2-14 hours of death. Tissue samples for toxicological analysis were placed in Whirl Paks® (Nasco, Modesto, California USA) and frozen immediately at –20°C, then transferred within 2-4 weeks to the University of Alaska Fairbanks, where they were stored at –80°C.

5.2.2 Heavy metals/minerals analyses of liver and kidney

Heavy metals and minerals analyses were performed on liver and kidney tissue. Samples were analyzed for Zn, Ag , Se, Cu and Cd at Texas A&M University, College Station, TX. Total mercury (tHg) analysis was conducted at University of Alaska Fairbanks. Previously published data (1983-1997, published in Woshner *et al.,* 2001) were combined with more recent data collected between 1998 and 2001. Detailed methods for Zn, THg, Ag , Se, Cu and Cd analysis can be found in Woshner *et al.,* (2001) and Dehn *et al.,* (2005a and b). QA/QC methods are covered in detail in Bratton *et al.,* (1997) and Dehn *et al.,* (2005 a and b).

5.2.3 Histology

Tissues were fixed in 10% neutral buffered formalin (NBF) at the time of sampling. Tissues were trimmed to the appropriate size and processed through graded alcohols to dehydration, passed through xylene to infiltrate with paraffin according to standard histologic procedure at the University of Alaska Fairbanks. Prepared tissue blocks were sectioned at 5 μm, placed on glass slides and stained with hematoxylin and eosin for general histological review. Select tissues (kidney and lung) were stained with Masson's trichrome stain for the assessment of collagen fibers (n=8). Prussian blue and Hall's bile stains were applied to selected liver tissue samples (n=8) to ascertain the type of pigment (i.e. hemosiderin) that was present. All stains were obtained and conducted according to methods provided by American Master*Tech Scientific (Master*Tech Scientific, Incorporated, Lodi, California, USA). Slides were then examined using light microscopy (Leitz Laborlux S, Leica Microsystems, Inc., Exton, Pennsylvania USA). Digital photomicrographs and measurements were taken using a Zeiss Axiocam camera and Axiovision software (version 3.2, Carl Zeiss, Inc., One Zeiss Drive, Thornwood, NY USA).

5.2.4 Tissue assessment/scaling methodology

Tissues listed in Table 5.1 were reviewed for each whale (n=64). In certain cases, collection of complete tissue sets was not possible due to conditions present at butchering or timing of arrival of necropsy crews. This research focuses on histological scoring in three main tissues of interest: kidney, lung and liver. Other tissues were reviewed and are covered in greater detail in a publication in process dealing specifically with histological findings in the bowhead whale (Rosa, unpublished data).

Areas of scored tissues described were chosen using a randomization procedure (Lovin field finder, Gurley Precision Instruments, Troy, New York USA) and were semi-quantitatively scored as described in Table 5.2.

5.2.5 Aging

Baleen stable isotope analysis of carbon ($\delta^{13}C$) signature and aspartic acid racemization of eye lens nuclei were used to estimate ages of 54/187 whales (George *et al.,* 1999; Rosa *et al.,* 2004; Lubetkin *et al.,* 2004). The remaining 133 whales were collected prior to our aging effort and were not included in analyes requiring age.

5.2.6 Statistical Analysis

Data are presented as the mean with standard deviation (SD). Desriptive statistical analyses were performed on the SAS operating system (SAS Institute, Inc., SAS Campus Drive, Cary, NC). Test statistics were considered significantly different at $P \leq 0.05$. Bonferroni adjustments were applied to maintain 5% significance levels. Interactions between metal/mineral concentrations and age, sex and season were determined using the general linear model (GLM) procedure in SAS. Simple linear regression was used to determine the significance of age on Zn, tHg, Ag , Se, Cu and Cd concentrations in the liver and kidney. Analysis of covariance (ANCOVA) was used to determine relationships between the dependent variables (Zn, tHg, Ag , Se, Cu and Cd), reproductive status and sex (female, male or pregnant female) and season (spring or fall) with age as a covariate. Canonical correspondence analysis (CCA) was used to examine the relationship between age, sex and season and element concentrations. Correlated metrics ($P \leq 0.05$) were removed prior to CCA to reduce probability of an arch effect (ter Braak, 1995). A global Monte Carlo permutation test was performed to test for existence of a relationship between age/season/sex and toxicant concentrations (ter Braak and Smilauer, 1998). A dimensionless toxicological concentration-age/sex/season biplot was constructed using CANOCO® to graphically examine the pattern of variation in element concentrations with respect to these variables (ter Braak 1995; ter Braak and Smilauer 1998). Biplot axes were interpreted by examining the sign and magnitude of their respective intra-set correlations and using subject-matter knowledge (ter Braak, 1995). Length of toxicant-specific eigenvectors (i.e., the arrows) in the biplot was interpreted as the strength of correlation between the metal/mineral concentrations and the variables (age/sex/season) (ter Braak, 1995). Therefore, long eigenvectors were most important with respect to the concentration of metals/minerals (ter Braak, 1995). Relative correlative ranking of toxicant levels with respect to variables was graphically represented by extending each eigenvector through the origin of the biplot and intersecting it with orthogonal lines drawn from the heavy metal/mineral of interest (ter Braak, 1995). Heavy metals/minerals positioned near the arrow- and blunt-end of the eigenvector were most positively and negatively correlated with the variable (age/sex/season), respectively (ter Braak, 1995). Cannonical correspondence analysis in CANOCO® does not provide univariate measures of association between individual metal/minerals and variables (ter Braak and Smilauer, 1998). We calculated Pearson's

coefficients of correlation and tested for a linear relationship between each toxicant and age (Milton and Arnold 1995). Ages that were correlated ($P \leq 0.05$) with metals/minerals also were regressed linearly using least-squares estimation, and univariate prediction models developed (Milton and Arnold, 1995). Metal and mineral concentrations were natural-log transformed for all correlation and regression analyses to satisfy ($P > 0.05$ as per Shapiro-Wilk test) linear model assumptions (Milton and Arnold, 1995).

5.3 Results

Summary statistics of mean heavy metals/minerals analyzed (Zn, tHg, Ag, Se, Cu, Cd) in bowhead whale liver and kidney are listed in Table 5.3. All results are expressed in μg/g wet weight. Metal correlation coefficients are listed in Tables 5.4 and 5.5. Age was significantly and positively associated with several of the elements (Table 5.6, Figures 5.2 and 5.3): Zn, Cd, and tHg in kidney and Zn, Cd, Se and tHg in liver. Season was found to relate to only one of the dependent variables: Se. Fall landed animals had a significantly higher concentration of Se in kidney tissue than spring collected animals (P=0.006, Spring: 1.28 ± 0.58, Fall: 1.50 ± 0.63). Elemental concentrations in liver and kidney did not vary by sex (Table 5.7).

Histologically, moderate to severe pulmonary fibromuscular hyperplasia was present in the terminal alveoli of many of the whales examined (grading scores 2 and 3, 17 of 40 whales examined). This occurred between the vessels in the septae; therefore, the epithelium/capillary relationship was not disturbed and air exchange appeared unaffected. With the exception of very young whales (< 5 years of age), whales commonly had pulmonary fibromuscular hyperplasia to varying degrees (Figure 5.4 (A) and 5.5 (A)), and it increased in magnitude with age.

In the kidney, moderate to severe thickening of Bowman's capsule and interstitial fibrosis (grading scores 2 and 3) were noted in many of the whales examined (21 of 63 whales examined, Figure 5.4 (B) and 5.5 (B)). Periportal hepatic fibrosis was mild when noted and occurred in nearly half of the whales examined (25 of 59 whales, Figure 5.4 (C)). These findings were analyzed separately with respect to age and Cd concentrations in the liver and kidney. Liver Cd concentration was strongly correlated with the degree of pulmonary fibromuscular hyperplasia (P= 0.001), moderately associated with degree of renal fibrosis (P= 0.03) and not associated with the degree of liver fibrosis (P= 0.06). Renal Cd concentration

was associated with the degree of pulmonary fibromuscular hyperplasia and renal fibrosis (each P=0.01), but not with the degree of liver fibrosis (P= 0.14). A significant age effect was found for both lung and renal fibrosis (P=0.001, $P \leq 0.0001$, respectively). With the exception of the above findings, there were very few lesions found in this sample set.

In the CANOCO® results, the global Monte Carlo permutation test based on 499 permutations revealed that the heavy metals/minerals investigated were associated (Liver: F= 6.603, P= 0.002, Kidney: F= 3.774, P= 0.02) with at least one of the variables investigated (sex, age, season, Figures 5.6 and 5.7). The first cannonical axis was most important in explaining variation in the concentrations of metals/minerals (i.e., as per respective eigenvalues). It collectively explained 71% (liver) and 75% (kidney) of variation in relative elemental concentrations. Intra-set correlations suggested that cannonical axis 1 explained most variation in metal/minerals concentrations with respect to age, because of the large absolute magnitude and strong relationship between Cd and age for axis one in both liver and kidney (Figures 5.6, 5.7, Table 5.8). The dimensionless biplot of axis one suggests that age was most positively correlated (as per eigenvector length) with the concentrations of elements analyzed. Sex/reproductive class was the next most strongly ranked determinant, though to a lesser extent than age. Season had little influence on the variables. Orthogonal inferred ranking of elements against eigenvectors indicated that Cd was positively associated with age in the liver and kidney, Hg was positively related to age in the liver and that Cd and Hg were weakly related to sex/reproductive class.

5.4 Discussion

Cetaceans have radiated from several distinct evolutionary lines (Sasaki *et al.*, 2005). These different lineages bioaccumulate essential and non-essential elements in different manners and exhibit different effects from exposure to toxicants (Dietz *et al.,* 1996; Muir *et al.,* 1999; Woshner *et al.,* 2001). In the Arctic, the odontocete species frequently studied include the beluga whale (*Delphinapterus leucas*) and the narwhal (*Monodon monoceros*). These whales occupy the top of the food chain, being mainly piscivorous, and are inappropriate for comparison to the bowhead whale, as it occupies a low trophic level, feeding on zooplankton. Bratton *et al.* (1997) and O'Shea and Brownell (1994) found that, in general, mysticete whales have lower concentrations of metals residues in their tissues than odontocetes, with the

exception of Cd. Other factors which may apply to both groups of cetaceans include large body size, low mass-specific metabolic rates, physiological and biochemical adaptations for deep diving, large storage compartments (blood, lipid), and wide amplitudes of seasonal cycles in fat storage and mobilization (O'Hara and O'Shea, 1999).

A key target organ of heavy metal toxicity is the kidney due to its remarkable ability to resorb and accumulate divalent metals (Barbier *et al.,* 2005a). In the present study, moderate to severe thickening of Bowman's capsule and interstitial fibrosis (grading scores 2 and 3) were noted in many of the kidney tissues examined histologically (21 of 63 whales). These changes were found to be age- and Cd-related; however, renal and hepatic Cd concentrations also showed an age-dependent increase. The present challenge is to determine if one or both of these factors (age, Cd concentration) are responsible for these observed differences. There were few whales in this study over the age of 60 years (n=5), but it is notable that renal Cd was found to be above average in all but one of these animals. The oldest whale (age estimate of 123 years) had a renal cadmium concentration lower than the average (14.51 $\mu g/g^{-1}$); unfortunately, we do not have a liver Cd value nor a histological sample from this animal for comparison. Plateaus and decreases in renal Cd concentrations in marine mammals of advanced age (using length as a proxy for age) have been reported recently (Dehn *et al.,* 2005a). The below average concentration found in this very old whale may be attributable to progressive kidney damage and sloughing of proximal tubule cells, though no evidence has been found of this histologically in other whales > 60 years in age. In the kidney, toxic levels of Cd affect proximal renal tubule function (Goyer, 1996; Beiglbock *et al.,* 2002; Takaki *et al.,* 2004). Morphological changes include tubular cell degeneration in the initial stages, progressing to an interstitial inflammatory reaction and fibrosis (Goyer, 1996). Proteinuria is one of the clinical signs of nephrotoxicity; it is principally tubular and irreversible. Additionally, the kidneys are particularly sensitive to oxidative damage and show more age-associated damage than other organs in the body (Percy *et al.,* 2005). All of these factors and the resultant loss of renal tubular cells could lead to decreased Cd concentrations in the renal tissues of older whales. Alternatively, as bowhead whales age, the area of the kidney that would normally contain functional renal tissue that concentrates Cd may be replaced by fibrosis. A present research priority involves intensively sampling older whales, as more information is needed to draw

conclusions regarding Cd burden in this cohort of animals. No evidence of renal insufficiency has been found in the whales examined via urinalysis and measurement of serum blood urea nitrogen and creatinine content (Rosa, unpublished data). Further investigation of kidney function is important, especially considering the hydration challenges [lack of fresh water, high salt load in prey (Nicol *et al.*, 1992)] that these animals have adapted to accommodate. Early microproteinuria may prove to be a useful marker in the diagnosis of Cd-induced nephrotoxicity (Bernard, 2004). It appears that the bowhead whale is able to tolerate even severe renal fibrosis and still function normally and produce young, as seems to be the case in other marine mammals that exist with high renal Cd concentrations (Deitz *et al.,* 1998; Rosa, unpublished data). Narwhal renal Cd concentrations are among the highest in marine mammals in the Canadian Arctic. Much of the cadmium (77%) in the renal cytosol is associated with metallothionein (Wagemann *et al.,* 1984). This form has been deemed to be relatively non-toxic. The "non-toxicity" of these thiol complexes has recently been brought into question, with research yielding preliminary results that may implicate these bound Cd-metallothionein moieties in the pathology of chronic renal damage (Barbier *et al.,* 2005b). Much of the Cd in marine mammal tissues is thought to be associated with MTH, and thus, a concurrent assessment of MTH in bowhead tissues is necessary for a complete overview of Cd dymamics and distribution in this species.

Moderate to severe fibromuscular hyperplasia was observed in the terminal alveoli of many of the whales examined (grading scores 2 and 3, 17 of 40 whales examined). Chronic obstructive pulmonary disease (COPD), pulmonary fibrosis and emphysema are health problems associated with chronic Cd toxicity in non-marine mammals. In the lungs, toxicity is proportional to time, level and route of exposure. Inhaled Cd induces the turnover and necrosis of alveolar macrophages. This releases enzymes that permanently damage the alveolar basement membrane, leading to rupture of septa and interstitial fibrosis. COPD develops secondary to chronic bronchitis, progressive fibrosis of the lower airways and accompanying alveolar damage leading to emphysema (Goyer, 1996; Stine and Brown, 1996). Route of Cd exposure is important and in terrestrial mammals is often via inhalation. The primary route of cetacean exposure is most likely gastrointestinal uptake (O'Hara and O'Shea, 2002). The effects of gastrointestinal Cd exposure on cetacean lungs require additional study. The fibromuscular hyperplasia noted in the lungs

of the bowhead whale has been related to Cd, but has also been found to be age-related. It does not appear to impede air exchange, and we speculate that this may be a normal developmental transformation for the species as diving experience increases, as it is seen to differing degrees in in all subadult and adult whales.

Hg in the environment comes from both natural and man-made sources. The mono-methyl form of mercury is of major toxicological significance as it enters the food chain and is ingested by animals, including marine mammals and man (Stine and Brown, 1996; Goyer, 1996; Caurant *et al.,* 1996). The tHg concentrations reported here are low in comparison to other cetaceans, which is consistent with previous studies (Bratton *et al.,* 1993; Woshner *et al.,* 2001). Concentration of tHg increases significantly with age; however, even in older whales (>60 years) it is over a magnitude lower than concentrations found in many piscivorous marine mammals (Krone *et al.,* 1999; Woshner *et al.*, 2001). Low trophic level and the species of prey consumed by bowhead whales are likely to account for these results, as the euphausid prey of bowhead whales is low in Hg (Honda *et al.,* 1983).

The various forms of Hg have different toxicities. The majority of Hg (50-100%) in muscle of marine mammals is in the organic form (i.e., methyl mercury), whereas the inorganic form makes up a higher proportion of the total Hg (70-90%) in the liver and kidney (Deitz *et al.,* 1999; Woshner *et al.*, 2001). Methyl mercury is important in terms of toxicity and health. Chronic mercury nephropathy has two phases: an early phase characterized by an anti-basement membrane glomerulonephritis followed by a superimposed immune complex glomerulonephritis with transiently raised concentrations of circulating immune complexes (Goyer, 1996). Rawson *et al.* (1995) noted lipofuscin accumulation in the livers of bottlenose dolphins (*Tursiops truncatus*) along with hepatic lesions (fat globules, central necrosis, lymphocytic infiltrates), though the pigment levels may be associated with age. Other studies investigating high (potentially toxic) levels of Hg have not shown mercury-specific lesions (Woshner, 2000; 2001), nor did we observe any in the present study (Rosa, unpublished data).

Se may play a crucial role in these circumstances. A potentially important relationship is thought to exist between Se and Hg in some marine mammals. Mercury levels that would be considered well above toxic levels in domestic animals are found in many marine mammals. These levels have been accompanied by increased selenium levels, often forming a 1:1 molar ratio between the two metals in certain species of

cetaceans (Koeman *et al.,* 1973; 1975). Each of these elements is thought to offset the toxicity of the other. It has been theorized that Se may act as an antioxidant in the glutathione peroxidase system, which reacts with free radicals and reactive oxygen species, which then affects the demethlyation of Hg. Once demethylated, the water-soluble Hg is trapped in the cell where it can bind other ligands (including sulfur and selenium). However, this 1:1 ratio was not noted in recent research involving beluga and bowhead whales, and more research is needed to characterize these interactions (Krone *et al.,* 1999; Woshner *et al.,* 2001; Dehn *et al.,* 2005a). These species may employ alternative methods to deal with increased concentrations of these elements.

Selenium was increase with age in bowhead liver, though this effect was not significant in the kidney. This is similar to findings in walrus (Wagemann and Stewart, 1994) and may be due to increased excretion of Se in the kidney of older animals or to the progressive renal fibrosis that is seen in bowhead whales of advanced ages (decrease in renal parenchymal tissue mass). Additional sampling of older individuals (>60 years of age) is needed to strengthen our assessment. Renal and hepatic Se has been found to increase with age in other cetacean species (Becker *et al.,* 1995; Woshner *et al.,* 2001), seals (Becker *et al.,* 1997; Wagemann *et al.,* 1996), and polar bears (*Ursus maritimus*) (Norstrom *et al.,* 1986; Braune *et al.,* 1991; Deitz *et al.,* 1999). Previous bowhead research showed that Se concentrations in both liver and kidney increased with length, which was being used as a proxy for age (Bratton *et al.,* 1997; Woshner *et al.,* 2001). Since ages were unknown in this earlier work, our aging techniques may account for the discrepancy in findings.

Most selenium is provided via diet and is considered essential mainly due to its presence at the catalytic sites of the enzyme glutathione peroxidase. This enzyme is involved in protecting cell membrane lipids and possibly proteins and nucleic acids from oxidant and free radical damage. The seasonal increase in renal Se noted in fall-landed whales has not been noted in previous studies, but may be related to the increase in feeding that occurs in summer and early fall. Questions as to the degree of Se absorption in bowhead whales, who are thought to employ some degree of forestomach microbial fermentation, exist (Herwig *et al.,* 1984) as ruminants are known to reduce selenide to an insoluble form in the rumen (Spears, 2003).

Previous marine mammal research has shown hepatic Se to have a strong positive correlation with Hg, Cd and Zn in the liver (Wagemann *et al.,* 1998). Significant correlations between Se and Cd and tHg in the liver and Se and Zn and Cd in the kidney were present in the bowhead tissues examined in this study. This is most likely due to the association between these metals and MTH.

Significant correlations between renal Se and the concentrations of Se, Cu, Cd, and Ag in the liver were detected. Selenium forms intracellular insoluble complexes with Ag, Cu, Cd and Hg, and may result in concurrent tissue accumulation of these metals along with symptoms of selenium deficiency. The requirement for selenium is related to the degree of oxidant activity and the supply of nutrients such as Zn, Cu, Vitamin E, Mn, and Fe. If these nutrients are increased, an increased need for Se will develop (Witchell, 1998; Van Metre and Callan, 2001). Many marine mammals that feed on upper trophic levels have considerable concentrations of these nutrients in their diet and additional selenium may be necessary for them to remain in the narrow range of adequate supply. Selenium requirements are unknown in large mysticetes. Selenium deficiency has been shown to cause cardiomyopathy and nutritionally-induced muscular dystrophy (white muscle disease) in terrestrial mammals (Witchell, 1998), and cardiomyopathy has been noted in *Kogia* spp. (Bossart et al., 1985). No clear connection between selenium levels and muscle necrosis or pathology has been noted in marine mammals, nor is it apparent in the bowhead whale (Rosa, unpublished data). However, this is a subject poorly understood in marine mammals and is in need of additional baseline research.

Copper is an essential element important for normal functions of hemapoietic and antioxidant systems (Goyer, 1996). Uptake is mainly via the gastrointestinal system and is regulated by body stores at the time of exposure. Cu was found in concentrations similar in range to those found in other cetaceans (Woshner *et al.,* 2001), and at levels lower than the normal range of values found in domestic mammals (Puls, 1994). Concentrations of Cu were not related to age, sex or season; however, there was a significant correlation noted between Cu and Zn in the liver and kidney and Cu and Ag in the kidney (Table 4). This association is likely to involve their relationship with MTH (Cu and Zn). Copper concentration in the kidney was significantly correlated with Ag in liver as well. Other significant interactions between liver

and kidney tissues included renal and hepatic Cu. This association is likely due to similarities in binding properties and distribution of this element.

Zinc is also an essential metal and is a critical cofactor for >200 metalloenzymes. While excess is generally not a problem, deficiency can lead to growth impairment, delay in sexual maturation and dermatologic problems in humans and domestic mammals (Goyer, 1996). Concentrations were similar to previously reported values in the bowhead whale and domestic mammals and do not appear to be of concern.

Zinc has shown to exert a protective effect in cases of heavy metal toxicity, especially in the case of Cd. Concurrent administration of Zn^{2+} during chronic Cd^{2+} toxicity has been shown to prevent the development of renal dysfunction, however, the mechanism of this protective effect is unknown (Barbier *et al.*, 2005a; Barbier *et al.*, 2005b). Zinc is known to interfere with heavy metal transport in renal cells, leading to a decrease in their resorption of these metals (Barbier *et al.*, 2005a; Barbier *et al.*, 2005b). Zinc levels were significantly associated with levels of Cu in the liver and Cu, Cd, Se and tHg in the kidney. This association is likely related to MTH.

Little is known about the presence of Ag and its dynamics and distribution in marine mammal tissues. A positive relationship was found between this element and Se, and it has been suggested that Ag, in the high concentrations found in beluga whales, may affect radical scavenging enzyme systems (Becker *et al.*, 1995a). However, in the bowhead whale, Ag was present in concentrations that were quite low in comparison to beluga whales and most domestic mammals and is unlikely to be a toxicant of concern in this species.

5.5 Conclusions

Age was related to many of the elemental concentrations determined, including Zn, Cd, Se and tHg. This emphasizes the effect of basic life history parameters on elemental concentrations. A significant association was found between the concentrations of renal and hepatic Cd and the amount of fibrosis found in the lung and kidney; however, both Cd concentration and fibrosis were also found to be strongly associated with age. Age is therefore a confounding or causative factor in these associations. These results highlight the importance of age as a variable in both toxicology research and histological assessment. The

determination of individual ages versus the use of length as a proxy for age in marine mammals will help to clarify some of these associations. Additionally, vigilance in combining element concentrations with quantifiable effects in tissues (via histology, biomarkers, etc.) will help elucidate the connection between toxicity and effects seen at the population level in these species.

5.6 Acknowledgements

We thank the Department of Wildlife Management, North Slope Borough (Barrow, Alaska), the Cooperative Institute for Arctic Research (CIFAR), UAF INBRE (Idea Networks for Biomedical Research Excellence) and NOAA for their generous funding and logistical support. We also thank the subsistence whalers in Barrow and Kaktovik and the Alaska Eskimo Whaling Commission (AEWC) for graciously allowing us to access these samples. Individual thanks to Gay Sheffield, Victoria Woshner, Lara Dehn, Mike Castellini, Doug DeMaster, Cyd Hans, Craig George, Benny Akootchook and Chris Terzi, for help with the manuscript, tissue collection and analytical support. Bowhead whale samples were collected under the authority of permit #932-1489-00 for the MMHSRP program (Dr. Teri Rowles). Support for this project came from CIFAR/NOAA (NSB BU3613) and Alaska INBRE grant number 2 P20 RR016466 from the National Center for Research Resources, National Institutes of Health, Dept. of Health and Human Services.

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Table 5.1. Key to tissues graded and analyzed histologically in the bowhead whale during the Inuit subsistence hunt in Northern Alaska (1998-2002). Autolyzed tissues are not included in the sample sizes listed below.*

Tissue collected	n
Kidney	63
Lung	40
Liver	59

* Other tissues collected, but not included in this analysis include: spleen, pancreas, brain, eye, tongue, trachea, adrenal gland, spinal cord, gastrointestinal tract (forestomach, stomach, duodenum, jejunum, ileum, colon), blubber, epidermis, pituitary gland, lymph nodes (from several regions), muscle, heart, bladder, testes, ovaries, uterus.

Table 5.2. Guidelines used during the histological scoring of bowhead whale tissues (kidney, lung and liver).

Kidney

Fibrosis

0	No fibrosis present
1	Mild fibrosis (glomerular wall measurements averages 3-10 um, collagen loosely arranged around glomeruli)
2	Moderate fibrosis (glomerular wall measurements averages 10-20 um, collagen loosely arranged around glomeruli
3	Severe fibrosis (glomerular wall measurements averages >20 um, collagen densely arranged around glomeruli)

Lung

Fibrosis

0	No fibrosis or muscular hyperplasia present
1	Mild fibrosis (1-10 um terminal alveolar width), minimal muscular hyperplasia
2	Moderate fibromuscular hyperplasia (1-20 um terminal alveolar width)
3	Severe fibromuscular hyperplasia (20-30 um terminal alveolar width)

Liver

Fibrosis

0	No fibrosis (walls of portal tracts <100 μm in thickness)
1	Mild periportal fibrosis (walls of portal tracts 100 to 150 μm in thickness)
2	Severe periportal fibrosis (walls of portal tracts > 150 μm in thickness)

Table 5.3. Elemental concentrations (μg/g-1 wet weight) in the liver and kidney of the bowhead whale collected in Barrow and kaktovik, Alaska (1983-2001). Means +/- standard deviation, range (in parentheses) and n are given.

Element	liver (μg/g-1 wet wt)	kidney (μg/g-1 wet wt)
Zn	36.11 ± 19.01 (6.99-135.11) n=110	26.90 ± 9.27 (9.07-56.31) n=108
Cu	9.81 ± 24.88 (1.09-203.81) n=117	2.39 ± 1.26 (0.76-7.94) n=108
Cd	7.85 ± 9.30 (0.03-50.91) n=117	16.47 ± 15.58 (0.01-64.00) n=108
Se	1.28 ± 0.71 (0.06-3.77) n=117	1.49 ± 0.45 (0.23-3.21) n=108
Ag	0.14 ± 0.33 (0.05-2.37) n=82	0.01 ± 0.01 (0.01-0.06) n=86
tHg	0.05 ± 0.05 (0.001-0.47) n=128	0.03 ± 0.03 (0.001-0.14) n=119

Table 5.4. Pearson's correlation coefficients for selected significantly correlated ($P \leq 0.05$) variables in liver and kidney of Alaskan bowhead whales collected between 1983 and 2001.

Liver	r	*P*	n	Kidney	r	*P*	n
Zn vs. Cu	0.44	<0.0001	119	Zn vs. Cu	-0.20	0.04	110
Cu vs. Ag	0.38	0.0004	84	Zn vs. Cd	0.59	<0.0001	110
Cd vs. Se	0.53	<0.0001	118	Zn vs. Se	0.25	0.009	110
Cd vs. Ag	-0.26	0.02	84	Zn vs. tHg	0.33	0.001	98
Cd vs. tHg	0.45	<0.0001	106	Cu vs. Ag	0.33	0.003	82
Se vs. tHg	0.51	<0.0001	107	Cd vs. Se	0.25	0.007	110
				Cd vs. tHg	0.48	<0.0001	98

Table 5.5. Pearson's correlation coefficients between liver and kidney concentrations of Zn, Se, Cu, Cd, tHg, and Ag in Alaskan bowhead whales collected between 1983 and 2001. Corellation coefficient, P value and n listed for each relationship.

Kidney	Zn	Cu	Cd	Se	Ag	tHg
Liver						
Zn	0.44	-0.16	0.24	-0.03	0.003	0.12
	<0.0001	0.105	0.015	0.762	0.978	0.251
	104	104	104	104	79	92
Cu	-0.03	0.23	-0.18	-0.32	-0.02	-0.15
	0.782	0.017	0.060	0.001	0.834	0.155
	104	104	104	104	79	92
Cd	0.32	0.09	0.80	0.24	-0.10	0.44
	0.0008	0.351	<0.0001	0.015	0.369	<0.0001
	104	104	104	104	79	92
Se	0.48	0.19	0.51	0.43	0.19	0.32
	<0.0001	0.053	<0.0001	<0.0001	0.095	0.002
	103	103	103	103	79	91
Ag	0.01	0.35	-0.28	-0.24	0.19	-0.24
	0.906	0.001	0.013	0.033	0.100	0.041
	79	79	79	79	79	68
THg	0.25	0.05	0.40	0.15	-0.08	0.45
	0.013	0.639	<0.0001	0.160	0.503	<0.0001
	94	94	94	94	70	111

Table 5.6. Simple linear regression results for significant relationships ($P \leq 0.05$) between selected elements in liver and kidney tissues with bowhead whale age (years) estimated via aspartic acid racemization or baleen isotopic analysis of $\delta^{13}C$.

Tissue	Element	n	r^2	*P* value
Kidney	Zn	26	0.181	0.0302
	Cd	26	0.381	0.0008
	tHg	26	0.379	0.0008
Liver	Zn	26	0.313	0.0030
	Cd	26	0.884	<0.0001
	Se	26	0.476	<0.0001
	tHg	26	0.314	0.0029

Table 5.7. *P* value statistics for the relationship between the sex class variable and hepatic and renal elemental concentrations.

Element	liver	kidney
Zn	0.22	0.06
Cu	0.53	0.09
Cd	0.90	0.32
Se	0.16	0.11
Ag	0.62	0.87
tHg	0.72	0.86

Table 5.8. Intra-set correlations of variables associated with the first two axes generated by a canonical correspondence analysis of elemental concentrations in the liver and kidney tissue of bowhead whales collected between 1983 and 2001 in Alaska.

Metric	Correlations[a]	
	Axis 1	Axis 2
Liver:		
Zn	-0.1767	0.0037
Cu	-0.3477	0.0383
Cd	0.4027	0.0001
Se	-0.2330	-0.1137
Ag	-0.3926	0.1960
tHg	0.2343	0.0351
Kidney:		
Zn	-0.0197	0.0586
Cu	-0.6239	-0.2133
Cd	0.8042	-0.1713
Se	0.0587	0.2251
Ag	-0.6002	0.2484
THg	0.1538	0.1090

[a] Intra-set correlations are standardized and dimensionless thus they can be interpreted as the strength and direction of life history characteristic-specific correlation and element concentration in the presence of all other metrics (ter Braak, 1995); we inferred that axis 1 (age) explained the majority of variation in elemental correlation because of the large absolute magnitude of Cd (liver) and Cd, Cu and Ag (kidney) for axis 1.

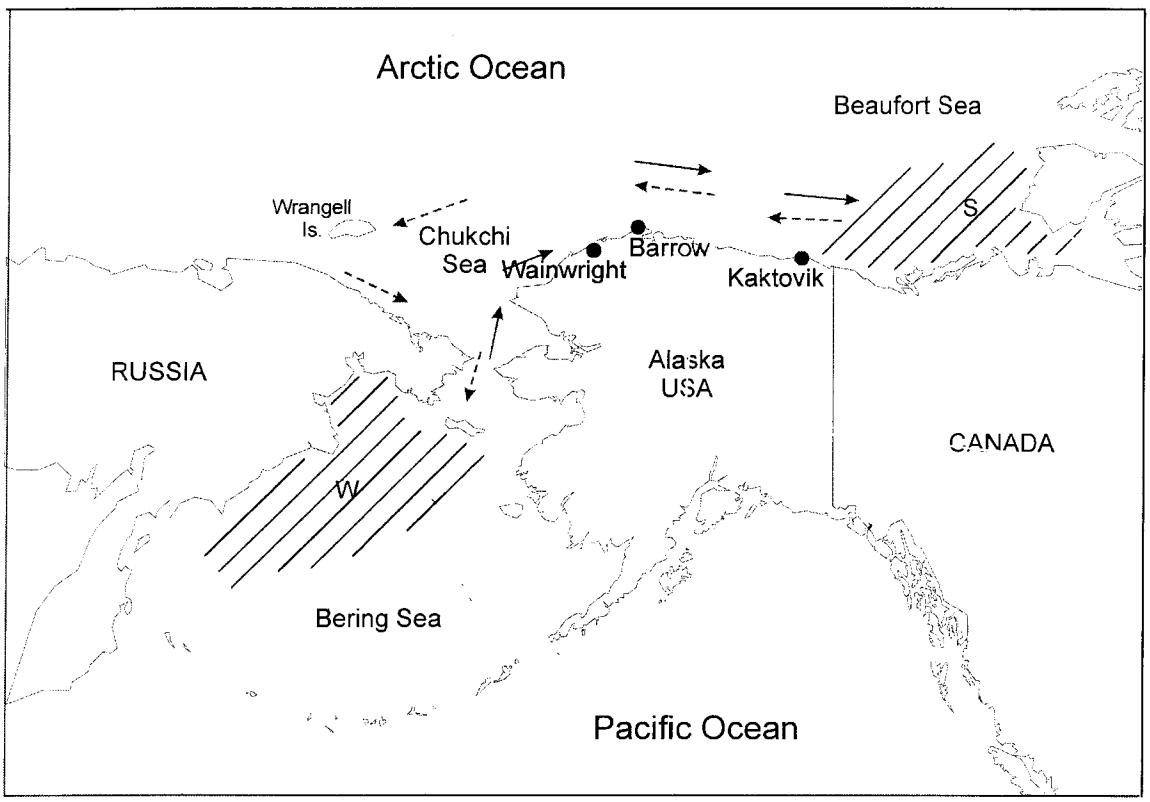


Figure 5.1. Bowhead whale sample collection occurred in Wainwright, Barrow and Kaktovik, Alaska between 1983 and 2001. (Modified from Hoekstra *et al.*, 2003). S=summer habitat, W=winter habitat.

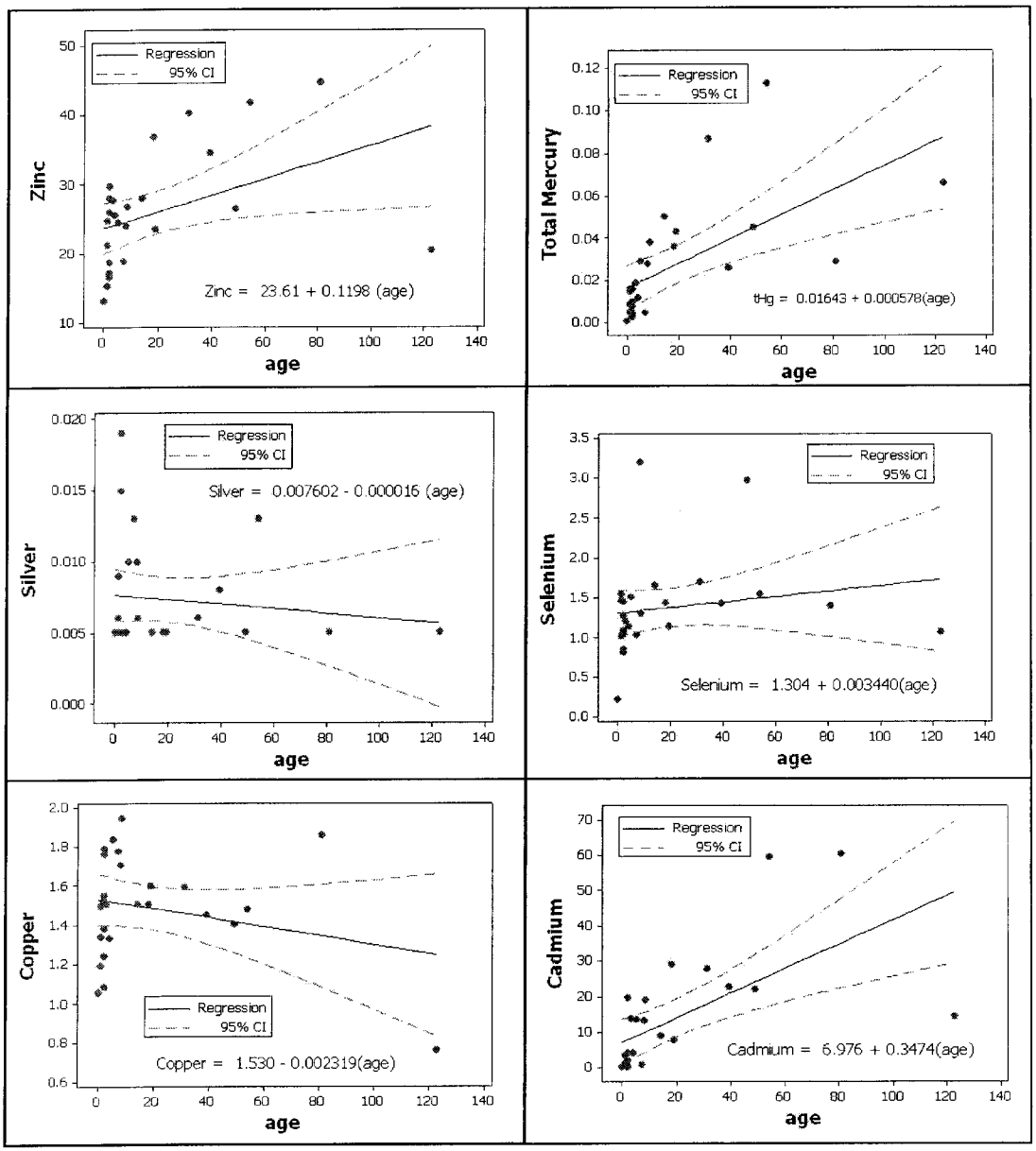


Figure 5.2. Concentrations of essential and non-essential elements in kidney tissue (μg/g wet mass) from the bowhead whale plotted against age (years) as determined by aspartic acid racemization of the eye lens nucleus or $\delta^{13}C$ istotopic analysis of baleen.

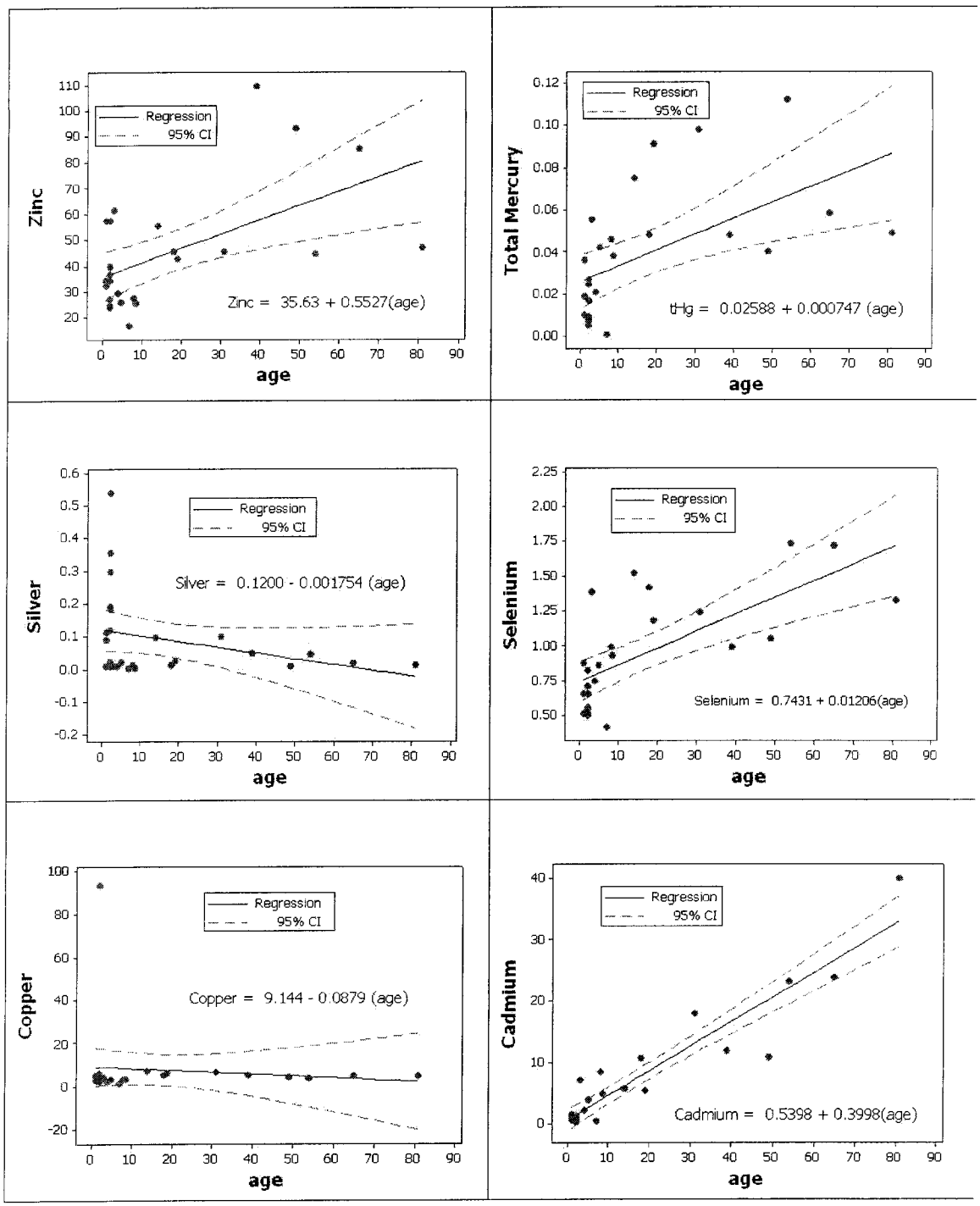


Figure 5.3. Concentrations of essential and non-essential elements in liver tissue (μg/g wet mass) from the bowhead whale plotted against age (years) as determined by aspartic acid racemization of the eye lens nucleus and $\delta^{13}C$ istotopic analysis of baleen.

(A)
Histomicrograph lung fibromuscular hyperplasia (four levels)
0 No fibromuscular hyperplasia present
1 Mild fibromuscular hyperplasia
2 Moderate fibromuscular hyperplasia
3 Severe fibromuscular hyperplasia

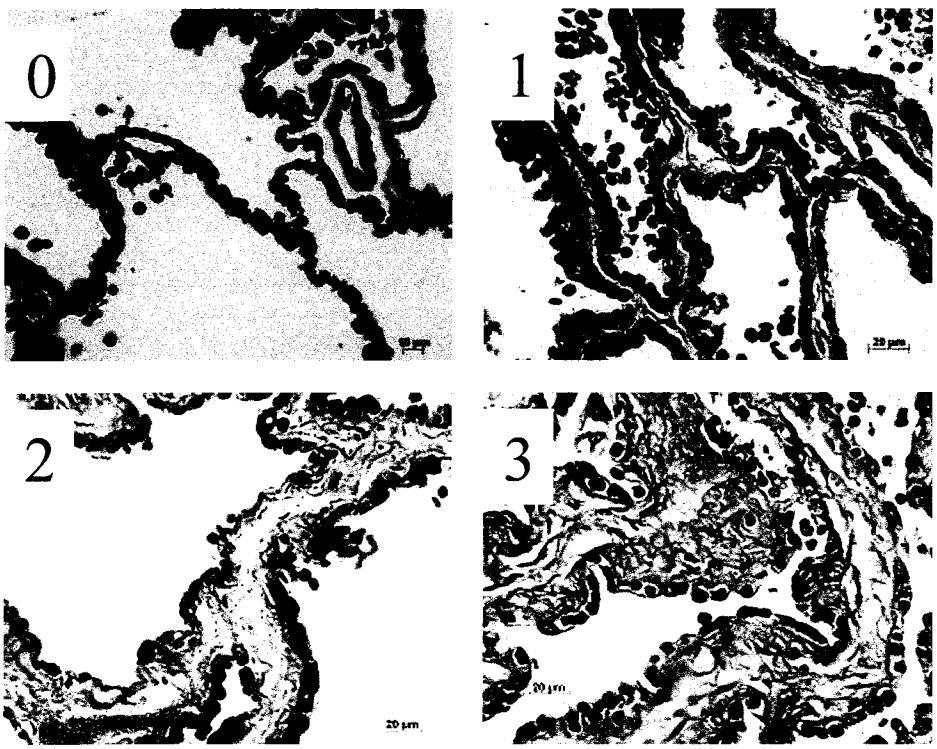
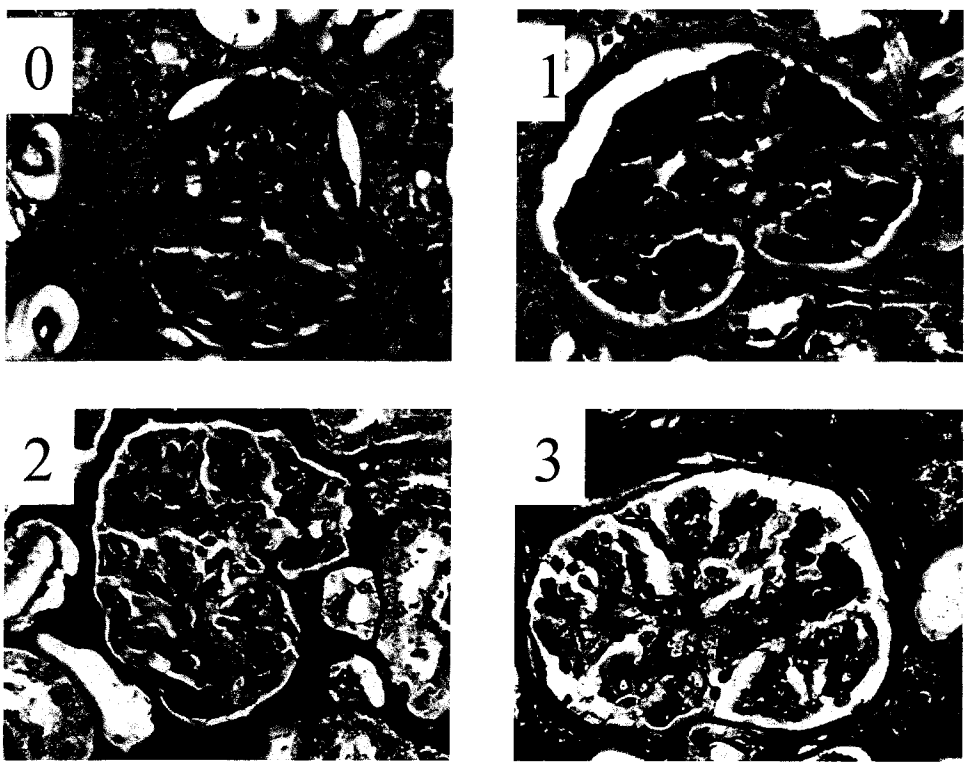


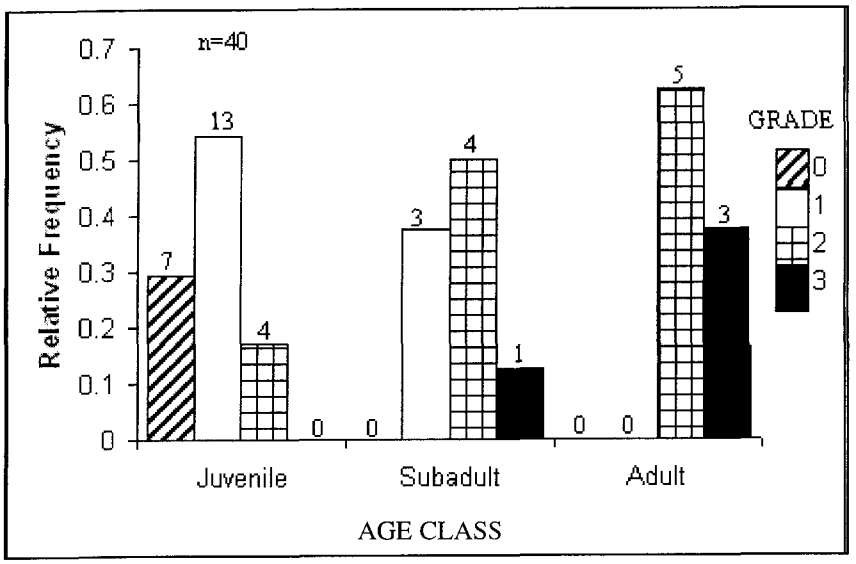
Figure 5.4. Photomicrographs of examples of the grades of histological change seen in (A) the lung and (B) kidney of the bowhead whale.

Figure 5.4. continued

(B)
Histomicrograph kidney fibrosis (four levels)
0 No fibrosis present
1 Mild fibrosis
1 Moderate fibrosis
2 Severe fibrosis



A. Lung: Fibromuscular hyperplasia severity by age class



A. Kidney: Fibrosis severity by age cohort

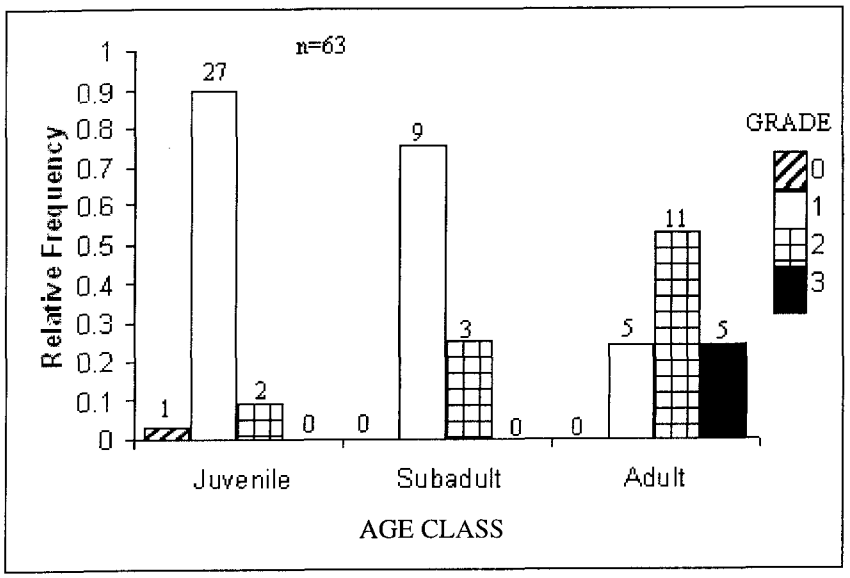
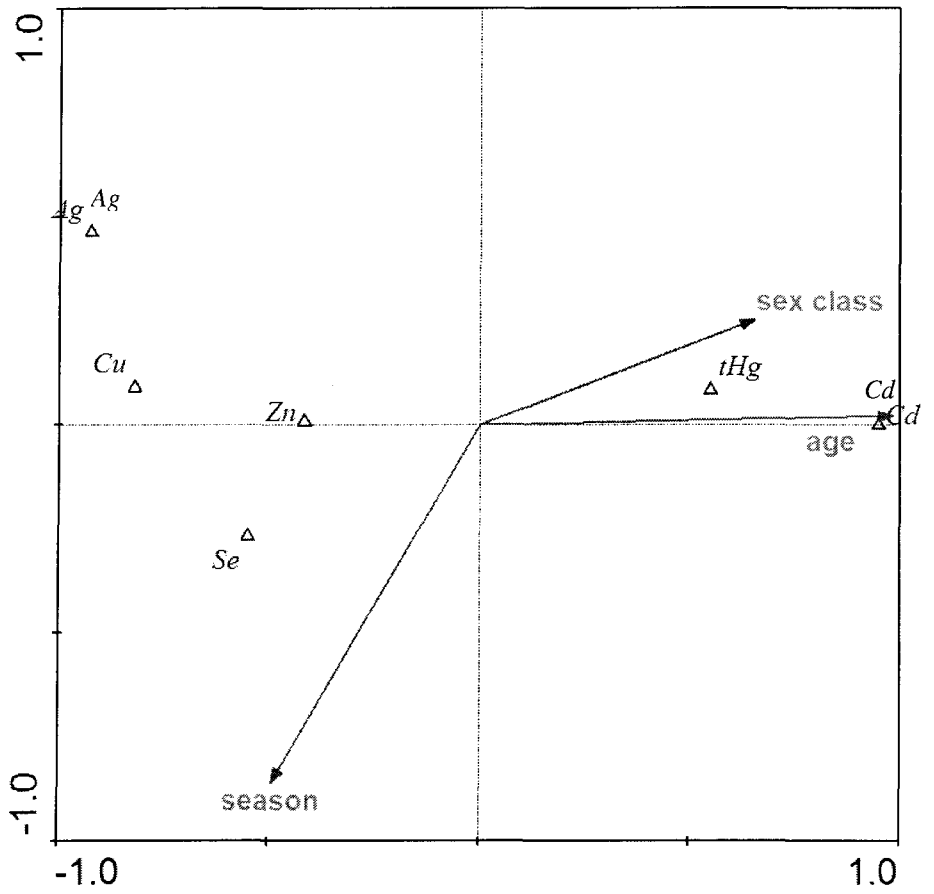


Figure 5.5. Relative frequency of each grade of scored (A) fibromuscular hyperplasia (lung) and (B) interstitial fibrosis (kidney) by age group in lung and kidney tissue from the bowhead whale. 0=little or no fibrosis, 1=mild fibrosis, 2= moderate fibrosis and 3= severe fibrosis (see Table 1 for scoring guidelines).

Figure 5.6. Liver CANOCO results in the bowhead whale. Canonical correspondence analysis of elemental concentration and life history characteristics (age, season of collection and sex) of the bowhead whale for samples collected between 1983 and 2001 in Alaska. (a) Elemental concentration biplot (ter Braak, 1995); axes 1 (ordinate) and 2 (abscissa) are dimensionless and represent age and sex, respectively (see corresponding intra-set correlations in Table 6); Length of eigenvectors (i.e. their respective eigenvalue) indicated the strength of the correlation between the variable and the pattern of variation in elemental correlation (ter Braak, 1995); elements more close to ends of eigenvectors are more positively correlated with it. (b) Inferred ranking of elements along variables based on biplot interpretation of Part a of Figure (ter Braak, 1995); ranking constructed by extending eigenvectors through the origin and intersecting with orthogonal lines from the element to the vector; the vertical segment represents the origin (i.e. centroid) of the biplot and is the grand mean of each variable; elements more close to the arrow or blunt end of are positively or negatively correlated with the variable, respectively.

Figure 5.6. Liver CANOCO results

A.



B.

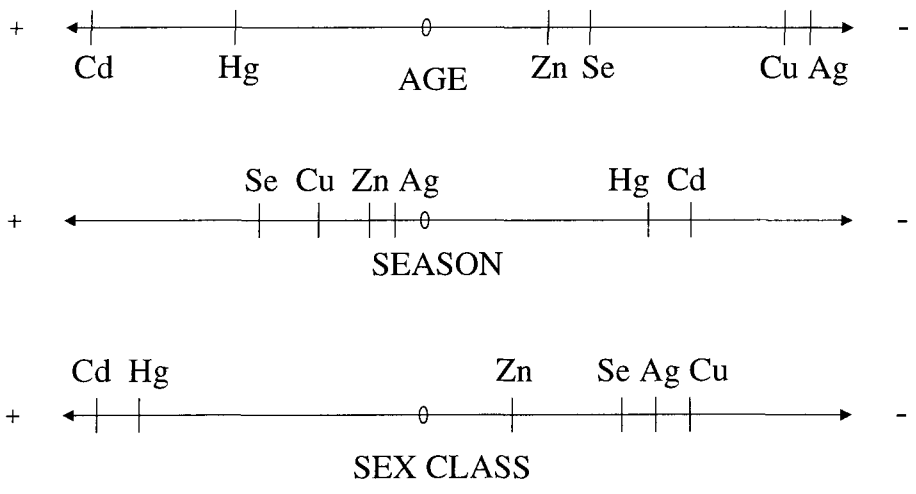
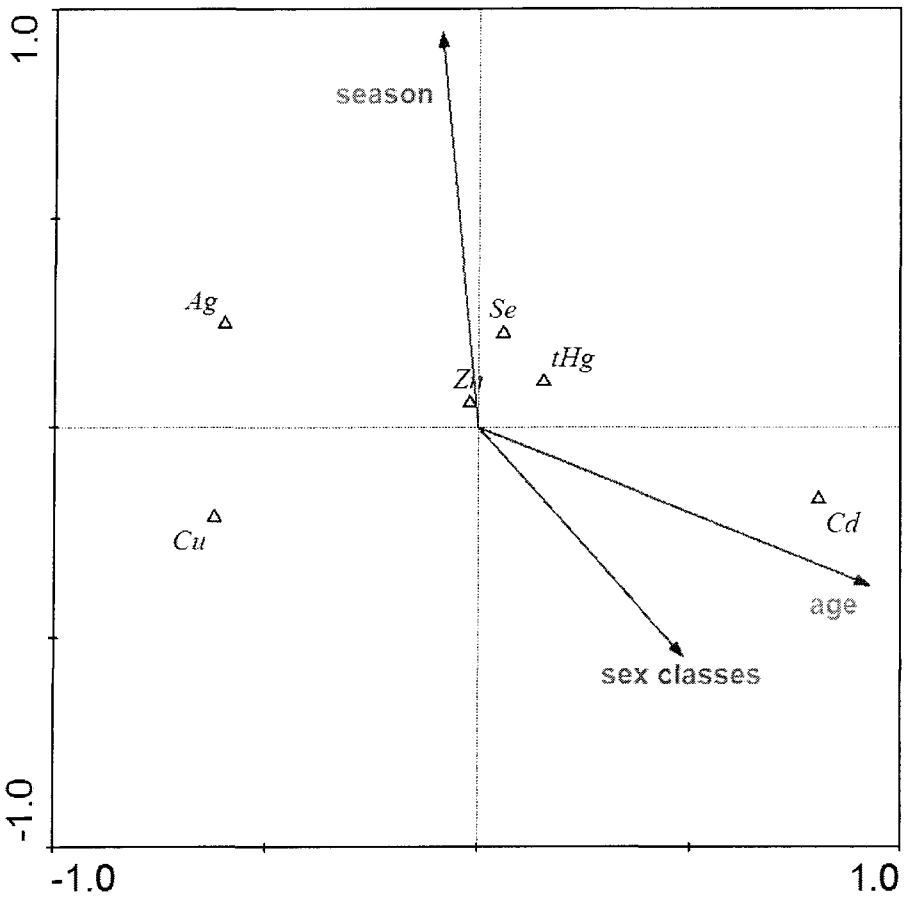


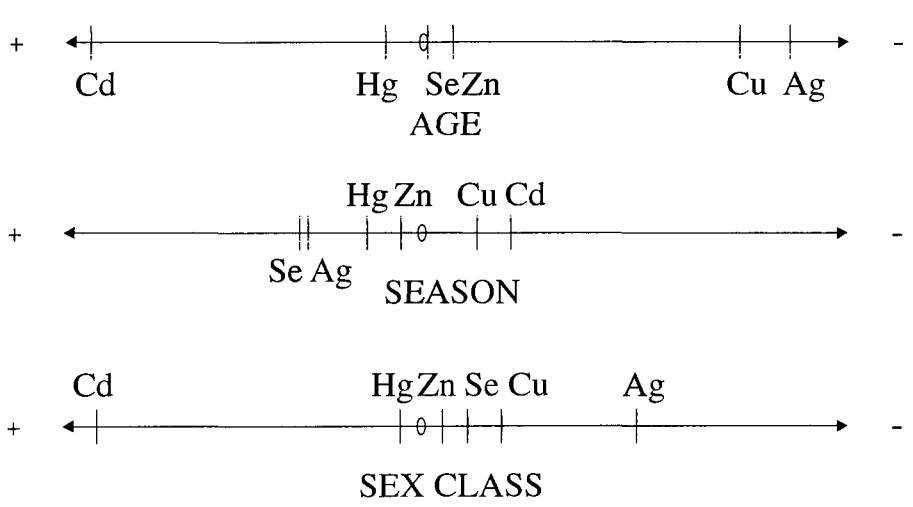
Figure 5.7. Kidney CANOCO results in the bowhead whale. Canonical correspondence analysis of elemental concentration and life history characteristics (age, season of collection and sex) of the bowhead whale for samples collected between 1983 and 2001 in Alaska. (a) Elemental concentration biplot (ter Braak, 1995); axes 1 (ordinate) and 2 (abscissa) are dimensionless and represent age and sex, respectively (see corresponding intra-set correlations in Table 6); Length of eigenvectors (i.e. their respective eigenvalue) indicated the strength of the correlation between the variable and the pattern of variation in elemental correlation (ter Braak, 1995); elements more close to ends of eigenvectors are more positively correlated with it. (b) Inferred ranking of elements along variables based on biplot interpretation of Part a of Figure (ter Braak, 1995); ranking constructed by extending eigenvectors through the origin and intersecting with orthogonal lines from the element to the vector; the vertical segment represents the origin (i.e. centroid) of the biplot and is the grand mean of each variable; elements more close to the arrow or blunt end of are positively or negatively correlated with the variable, respectively.

Figure 5.7. Kidney CANOCO results:

A.



B.



Chapter Six

Renal interstitial fibrosis, pulmonary fibromuscular hyperplasia and other findings from a histological assessment of the bowhead whale (*Balaena mysticetus*)[5]

6.0 Abstract

We performed gross examinations and collected tissues for histological assessment during the Inuit subsistence harvest of bowhead whales (*Balaena mysticetus*) in Northern Alaska. Tissue samples were collected for histological analyses from bowhead whales (n=64) hunted during the spring and fall in Barrow and Kaktovik, Alaska (1998-2002). Our objectives were to describe the range of normal histological findings in the species and to define the prevalence of disease in the Bering-Beaufort-Chukchi Sea stock of bowhead whale. We identified and discriminated abnormalities that could be attributed to heavy metal/mineral toxicity, specific disease entities, age, reproductive status or capture. Overall, few pathological changes were observed during gross necropsy or histological assessment. Qualitative observations were made more quantitative through the use of histological staining, digital imaging/measurement and rating profiles, which allowed the assignment of histological observations to a clearly-defined scoring system.

Abnormalities were few, consisting mainly of hepatic and renal fibrosis and pulmonary fibromuscular hyperplasia. Additionally, pigment was observed in the liver (25/58 whales examined) and extra-medullary hematopoeisis was noted in the spleen (17/45 whales examined). The putative effects of seasonal feeding and fasting on the pancreas and liver were assessed through the evaluation of pancreatic zymogen stores and the degree of hepatic lipidosis observed. Minimal parasitism was noted.

Keywords: Arctic, bowhead whale, disease, heavy metals, histology

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[5] Rosa, C., O'Hara, T.M., Gray. M.J., Blake, J.E. Renal interstitial fibrosis, pulmonary fibromuscular hyperplasia and other findings from a histological assessment of the bowhead whale (*Balaena mysticetus*). Prepared for submission to the Journal of Cetacean Research and Management

6.1 Introduction

Many of the world's cetacean populations are being impacted by disease and anthropogenic stressors in their environment (Martineau *et al.,* 1994; De Guise *et al.,* 1995; Moore *et al.,* 2004; Gulland *et al.,* 2005). As a result, population health assessment has become a more prevalent part of cetacean research (Busbee *et al.,* 1999; Pettis *et al.,* 2004; Gulland *et al.,* 2005). Histological interpretation of tissue samples is an important component of these efforts; therefore, establishing a baseline histological profile of each cetacean species is critical to interpreting pathological changes that affect individuals or the population. Obtaining well preserved tissues from large mysticetes that are suitable for high quality histological sections is difficult. Mysticetes are often impossible to keep in captivity and present logistical challenges when they are landed or recovered after a mortality event due to their large size and conditions surrounding necropsy. Additionally, many of the tissues collected from cetaceans are from stranding events. Cetacean strandings are often related to illness or trauma (Wilson *et al.,* 1997; Bossart *et al.,* 2003; Moore *et al.,* 2004), and samples collected from stranded individuals may not be representative of the population. Obtaining tissues suitable for histological fixation from animals that are in normal health is critical for full evaluation and comparison of the gradient for health and disease states.

Tissues gathered and preserved in a timely fashion after death will minimize the incidence of autolytic change and result in high quality samples for review. Timely collection can be problematic with respect to stranding events (Moore *et al.*, 2004). Publications on the histology of large cetaceans are limited, but a recent review of necropsy findings in right whales (*Eubalaena glacialis*) stranded on the east coast of the United States includes necropsy findings from 18 adults and juveniles and 12 calves. Autolysis secondary to extended periods of time elapsing before carcass discovery and slow tissue recovery time resulted in many of the collected tissues being unsuitable for histological interpretation (Moore *et al.,* 2004). Sample size is another consideration when determining baseline values. Several detailed anatomical studies of specific systems have been performed on the bowhead whale (*Balaena mysticetus*), providing a sound foundation for the effort presented here (Albert, 1981; Haldiman *et al.,* 1984; 1985; Henk *et al.,* 1986; 1990; 1996). However, these findings were often taken from a small sample of whales and were not interpreted with respect to the age of the animal, gender, season of collection or heavy metal/mineral status.

These comparisons are important to the interpretation of histological findings, as changes may occur normally with respect to these variables. Such expected changes must be documented for proper interpretation of histological findings. This process is enhanced by the development of a means of quantification of histological findings. Many new technologies exist for the quantitative grading of histological change. Digital imaging, coupled with special stains and image analysis of randomized samples, helps to reduce some of the subjectivity present in qualitative histological assessments.

The bowhead whale is an endangered arctic mysticete. The Bering-Chukchi-Beaufort Sea (BCBS) Stock was commercially exploited in the mid to late 1800's. Today, the population has recovered to more than 10,000 individuals and is growing (George *et al.,* 2004). The bowhead whale is an important subsistence species to residents of Northern Alaska, Russia and Canada. The BCBS stock migrates yearly between the Bering Sea and the Beaufort Sea. The Beaufort Sea is currently used for offshore oil exploration (i.e., seismic testing methods) and production and may be experiencing significant climate change effects (Hinzman *et al.*, 2005). This has increased interest in the establishment of a baseline health profile for bowhead whales and monitoring of their population health. The Inuit subsistence hunts provide the opportunity to obtain high quality biological samples in the spring and fall.

This study describes the unique histological and histopathological findings from bowhead whale tissues collected during the Inuit subsistence hunt in Barrow, Alaska between the years 1998 and 2002. Additionally, we have investigated the relationship between these histopathological findings and age, season, sex, and heavy metal/mineral status.

6.2 Materials and Methods

6.2.1 Sample collection

Morphometric measurements (Appendix 6.1) and a suite of tissues (for histological, toxicological and hormonal analyses) were collected from bowhead whales (n=64) during the 1998-2001 spring and fall Inuit subsistence hunt in Barrow and Kaktovik, Alaska (Table 6.1). Scientific collection was conducted with permission from the Barrow and Kaktovik Whaling Captains Association and the Alaska Eskimo Whaling Commission (AEWC) through the North Slope Borough Department of Wildlife Management (NSB-DWM) under the purview of a National Oceanic and Atmospheric Administration (NOAA) permit

issued to Dr. Teri Rowles (#932-1489-00 for the Marine Mammal Health and Stranding Response Program). Whale examination crews followed standard NSB-DWM tissue collection protocols for bowhead whales landed (generally 3-14 hours after death). A standard histological collection list is included in Table 6.1. The position of the whale during butchering, extensive butchering by the hunters prior to arrival of sampling crew or dangerous ice conditions precluded collection a complete set of tissues from some whales. Also, tissues that were autolyzed or frozen, and therefore not appropriate for histological analysis were not included in the data set. Age was calculated via a combination of aspartic acid racemization of the eye lens nucleus and/or stable isotope analysis ($\delta^{13}C$) of baleen (George *et al.*, 1999; Rosa *et al.*, 2004; Lubetkin *et al.*, 2004). Animals 1-3 years of age were classified as juveniles, males from 4-22 years of age and females 4-25 years of age were classified as subadults and whales >22 years of age (males) and >25 years of age (females) were classified as adults (Zeh *et al.*, 1993; George *et al.*, 1999).

Tissues were fixed in 10% neutral buffered formalin (NBF) at the time of sampling. Tissues were trimmed to the appropriate size, processed through graded alcohols to dehydration, and passed through xylene to infiltrate with paraffin according to standard histologic procedure at the University of Alaska Fairbanks. Prepared tissue blocks were microtomed at 5 μm, placed on glass slides and stained with hematoxylin and eosin. Select tissues of interest (kidney and lung) were also stained with trichrome stain for the assessment of collagen fibers (n=8 whales). Prussian blue and Hall's bile stains were applied to selected liver tissue samples (n=8 whales) to ascertain the type of pigment (i.e., hemosiderin) that was present. Slides were then examined using light microscopy (Leitz Laborlux S, Leica Microsystems, Inc., Exton, Pennsylvania USA). Digital photomicrographs and measurements were taken using a Zeiss Axiocam camera and Axiovision software (version 3.2, Carl Zeiss, Inc., One Zeiss Drive, Thornwood, NY USA).

6.2.2 Tissue assessment/scaling methodology

Areas of scored tissues described were chosen randomly (Lovin field finder, Gurley Precision Instruments, Troy, New York USA) and were semi-quantitatively scored as described in Table 6.2.

Heavy metals/minerals analyses of liver and kidney

Analyses of heavy metals and minerals were performed on liver and kidney in the present study. Detailed methods for Zn, THg, Ag, Se, Cu and Cd analysis can be found in Woshner *et al.* (2001) and Dehn *et al.*(2005a and b). QA/QC methods are covered in detail in Dehn *et al.* (2005 a and b).

6.2.3 Statistics

The Chi-square test of homogeneity was used to test for difference in relative frequency distributions of renal fibrosis and pulmonary fibromuscular hyperplasia among ages. Cochran-Mantel-Haenszel statistics were used to test association between variables (i.e. pulmonary/renal fibrosis and renal/hepatic Cd concentrations) and to test the relationship between age/sex/season and the mean score of response on the histological scoring chart. Analysis of covariance (ANCOVA) was used to determine relationships between the dependent variable (Cd), reproductive status and sex (female, male or pregnant female) and season (spring or fall) with age as a covariate.

6.3 Results

We examined 64 bowhead whales harvested by Inuit hunters during the spring and fall migrations in 1998 through 2002. We made full tissue collections made for glass slide sets for 21 whales and partial slide sets for 43 whales. Descriptions of light microscopic anatomy are limited to normal anatomy that is considered unusual when compared to terrestrial species and to abnormal changes that are liable to affect interpretation of bowhead whale histology.

6.3.1 Description of light microscopic anatomy

Kidney

The kidney in the bowhead whale was composed of clusters of renicules, each of which has its own individual cortex, medulla and renal papilla. The cortex surrounds the inner medulla which contains pyramids and lacks glomeruli. The cortex contains renal corpuscles that contain glomeruli. The glomeruli are tuft-like in appearance and are composed of lobules of capillaries that arise from afferent arterioles and drain into efferent arterioles. No evidence of glomerular basement abnormalities was seen in any of the reviewed samples (n=63). Mesangial cells and podocytes are present. Medullary rays are present in the cortical region, containing the collecting tubules and the loops of Henle. The proximal tubules have microvilli present on their internal border and are lined with cuboidal to columnar epithelium. The

juxtaglomerular apparatus is present at the base of the glomerulus and consists of modified smooth muscle cells and the macula densa, which is made up of tall, cuboidal cells.

Moderate to severe thickening of Bowman's capsule and interstitial fibrosis (grading scores 2 and 3) were noted in a third of the kidney tissues examined (21 of 62 whales examined, Figure 6.1, a-d). These findings were analyzed with respect to age and Cd concentrations in the liver and kidney (results follow below).

Spleen

The spleen had a thick capsule (500-600 μm) composed of dense fibro-elastic tissue. We observed no additional supporting tissue or internal trabeculae in the bowhead whale spleen. The splenic parenchyma is composed predominantly of red pulp that appears to be organized as splenic cords and sinuses. Distinct, organized white pulp was not observed in any of the 49 whales we examined. Organized lymphoid follicles and periarteriolar sheaths were absent. Multifocal, mild to marked, extramedullary hematopoiesis, with prominent megakaryocytes was noted in 37% (17/45) of samples reviewed (Figure 6.2).

Pancreas

The exocrine pancreas appears strongly basophilic. Each acinus is composed of an irregular cluster of pyramid-shaped secretory cells. These cells form apically-oriented secretory complexes with zymogen granules present along the luminal border of >95% of animals examined (Figure 6.3). Centroacinar cells with pale cytoplasm and pale nuclei are present in the center of these acini. Acini are separated by a small amount of connective tissue and numerous capillaries. Zymogen status was evaluated in pancreases reviewed. In general, zymogen status was good to excellent (based on the amount of zymogen present and the stain intensity present in the granules) in all but one of the whales, with whales exhibiting plentiful, strongly-acidophilic zymogen granules. Only one of twenty-eight whales examined appeared to have a degree of zymogen depletion (mild to moderate). This individual was a subadult male harvested in the fall that had no gross or histological abnormalities noted that were atypical of the other whales in this sample (i.e., mild renal fibrosis and pulmonary fibromuscular hyperplasia were present, but lymph nodes and other tissues examined were normal). Girth measurements were similar to other whales in his length range.

The endocrine pancreas consists of the islets of Langerhans, which are round, compact and appear to be highly vascularized with little associated connective tissue. The cells that compose the islets are small with a poorly-stained, granular cytoplasm.

Lung

The lung parenchyma consists of bronchi, bronchioles and alveoli. The bronchi are lined with ciliated, pseudostratified, columnar epithelium. Goblet cells are common (Figure 6.4a). Discrete cartilagenous foci are present in small, medium and large airways. Septal walls have double capillary beds typical of cetaceans. Moderate to severe fibromuscular hyperplasia was present in the terminal alveoli of many of the whales examined (grading scores 2 and 3, 17/40). This occurred between the vessels in the septae; therefore, the epithelium/capillary relationship was not disturbed and air exchange appeared unaffected. With the exception of very young whales (< 5 years of age), whales commonly had this finding to varying degrees and it increased in magnitude with age (Figure 6.4, a-d).

Liver

The bowhead whale liver has a prominent fibrous capsule. Central veins are present surrounded by hepatic cells, sinuses and portal triads. Hepatocytes are polygonal with abundant granular and esosinophilic cytoplasm. They possess central round to oval-shaped nuclei. Some pleomorphism and occasional binucleate hepatocytes were noted. "Plates" of hepatocytes are lined by sinusoids which are interposed between these plates. Portal triads are demarcated by fibrous connective tissue and contain branches of the hepatic artery, portal vein and intralobular bile ducts. Intralobular bile ducts are lined with low cuboidal to cuboidal epithelium. Bile ductules are not visible and Kupfer cells were not prominent via light microscopy. Mild periportal fibrosis was present in nearly half of the whales examined (25 of 59 whales examined). Lipocytes (or possibly Stellate (Ito) cells) were present in most bowhead livers (47 of 59 whales examined) Additional methods (immunohistochemistry) are needed to discriminate between these cells. Golden-brown to dark-brown pigment was noted in 42% (25/59) of the livers examined. This pigment was identified as hemosiderin via special histological staining (Figure 6.5, a-c).

6.3.2 Variables associated with histologic findings

Liver Cd concentration was strongly correlated with the degree of lung fibrosis (P= 0.001), and renal fibrosis (P= 0.03) but did not significantly correlate with the degree of liver fibrosis (P= 0.06). Renal Cd concentration was correlated with the degree of lung and renal fibrosis (P=0.01, 0.01, respectively), but not the degree of liver fibrosis (P= 0.14). A significant age correlation was found for both lung and renal fibrosis (Figure 6.6, a and b). A significant correlation was also found between age and Cd in both liver and kidney (liver: n=26, R^2=0.884, P=<0.0001; kidney: n=26, R^2=0.381, P=0.0008) (Figure 6.7, a and b). There was no relationship found between the presence/amount of hepatic pigment, fibrosis or lipidosis and age, sex or season. There was no age or seasonal effect found on the degree of EMH in the spleen. Additionally, no seasonal association was noted with respect to presence or absence of pancreatic zymogen.

6.4 Discussion

In this study, we focused on five organs in the bowhead whale: kidney, spleen, pancreas, lung and liver. These tissues have a high potential for health-related histologic changes of interest to occur and were frequently observed with histopathology. These organs are easily located, identified and sampled and are commonly collected as a part of routine health assessment protocols. A full characterization of the concentration of heavy metal/minerals in the kidney and liver tissue of these whales is covered in another work currently in progress and in previous publications (Woshner *et al.*, 2001; Dehn *et al.*, 2005b).

The health effects of chronic exposure to heavy metals such as lead, cadmium, and mercury are widely documented, yet few data exist on the histological impact of Cd exposure on renal histology in marine mammals (Bergman *et al.*, 2001; Sonne-Hansen *et al.*, 2002; Woshner *et al.*, 2002). Renal interstitial fibrosis was one of the most striking findings in this study. This was of interest as renal cadmium concentrations of bowhead whales are relatively high in comparison to some cetaceans and terrestrial mammals (Bratton *et al.*, 1993; Woshner *et al.*, 2001, Dehn *et al.*, 2005). In previous laboratory and wild mammal studies, Cd has been associated with renal and pulmonary fibrosis (Aughey *et al.*, 1984; Goyer *et al.*, 1989; Beiglbock *et al.*, 2002; Damek-Poprawa and Sawicka-Kapusta, 2003). In bowhead whales, a significant age association was found with respect to the degree of observed renal fibrosis; however, both hepatic and renal concentrations of Cd were also found to correlate with the degree of fibrosis noted via

quantitative histological grading. Hepatic and renal Cd concentrations also increased linearly with age in this study and have previously been reported to increase with increasing body length (Woshner *et al.,* 2001; Dehn *et al.,* 2005b). It is difficult to separate age and Cd bioaccumulation as factors associated with fibrosis. These two factors are clearly not independent from one another in bowhead whales (Woshner *et al.* 2001; Dehn *et al.,* 2005b). Long-term cadmium exposure has been found to accelerate aging changes in the kidneys of laboratory rodents (Heidland *et al.,* 2001; Percy *et al.,* 2005). Previous non-cetacean research has shown accumulation of renal extracellular matrix (ECM) proteins to be progressive and related to end-stage kidney disease in humans and laboratory rodents (Verbeke *et al.,* 1997; Heidland *et al.,* 2001). However, recent research has shown differences between the accumulation of protein matrix (fibrosis) that occurs with aging versus that which occurs in disease states in the kidney (Abrass *et al.,* 1995). The three major age-related changes found in the kidney of laboratory rodents involved thickening of the glomerular basement membrane, increased ECM within the renal interstitium and focal areas of tubule atrophy. These findings are similar to features observed in the bowhead whale, with the exception of tubular atrophy. No other significant pathology (i.e., proximal tubule necrosis, glomerular epithelial cell hypertrophy, tubulo-interstitial nephritis) was noted in the bowhead whale kidneys examined. Despite the severe interstitial fibrosis found in several whales in this study, there is no indication that there had been an adverse effect on kidney function. Assessment of renal function is logistically difficult in this species; however, serum blood urea nitrogen, creatinine and urine specific gravity values analyzed from a subsample of these whales were normal in comparison to other cetacean and mammalian reference ranges (C. Rosa, unpublished data). We speculate that the majority of the fibrotic changes observed are secondary to natural aging processes; however, this cannot be demonstrated with full certainty. A more complete understanding of collagen deposition in the bowhead whale would help clarify the relationship between age-related change and pathological change in the kidney, as research in other species has shown increased renal collagen accumulation and new expression of matrix proteins in response to specific disease processes (Abrass *et al,* 1995).

None of the whales examined exhibited underlying disease processes that would necessitate the need for hematopoiesis outside of the marrow cavity, yet 37% of these whales exhibited mild to marked

extramedullary hematopoiesis (EMH). EMH has been reported in spleens of clinically normal mice of all ages and also in various tissues of clinically normal common cotton-eared marmosets (*Callithrix jacchus*, Okazaki *et al.,* 1996). We found no documentation of EMH in marine mammals in the literature. This phenomenon may be a normal or diet-related occurrence in bowhead whales. A rodent-based study documented increased hepatic brown pigment accumulation and splenic EMH in mice fed fish oil on a long-term basis (Steerenberg *et al.,* 2002). The bowhead whale diet is composed mainly of euphausids, amphipods, copepods, and mysid shrimp (Lowry, 1993; Richardson and Thomson, 2002) which are high in omega fatty acids (Ruggiero-Lopez *et al.,* 1994) and could cause similar changes. As an alternative hypothesis, an osteopetrosis-like syndrome may occur in these large whales. The bones of adult whales have been noted to be remarkably dense in comparison to terrestrial mammals by the author. This may be, in part, to promote balanced buoyancy for their blubber-laden body. The marrow cavity in adult whales may be diminished to the point that EMH is necessary for red cell production. It is possible that additional areas of red blood cell production are required to meet the hematopoietic needs of this species (blood which is high in hemoglobin and muscles that are rich in myoglobin which are diving-related adaptations) (Reynolds and Rommel, 1999). These needs may also vary seasonally, or with life history events, however, age and season of harvest did not affect the presence or amount of EMH observed in the bowhead whale. More investigation into diet, red blood cell production and bone marrow of large cetaceans is warranted, as well as attention to the presence of EMH in other species of mysticetes.

The pancreatic acini in 27 of the 28 pancreatic samples examined were remarkably zymogen-rich. Starvation and malnutrition have been shown to induce zymogen depletion in non-fasting adapted animals (Godet *et al.,* 1983; Kitagawa and Ono, 1986; Nagy *et el.,* 1989; Mizushima *et al.,* 2004). This scenario has not been investigated in marine mammals that are physiologically adapted to fasting, though there has been one report relating zymogen depletion to chronic malnutrition in a gray whale (Dailey *et al.,* 2000). Unfortunately, due to the enzymes present within the organ, pancreatic tissue deteriorates rapidly after death, making collection of pancreatic samples appropriate for histologic review rare in stranded cetaceans. All pancreatic tissue in our sample had a normal gross appearance. No seasonal variation in zymogen content was found. This is of interest, as bowhead whales are thought to fast for several months during the

year (Lowry, 1993), yet they appear to accommodate fluctuations in forage availability with minimal gross or histological effect on the pancreas. This likely indicates adequate caloric reserves to sustain them throughout their annual fasting period; however, the lack of seasonal differences in zymogen status may not be as relevant if bowhead whales do not fast as extensively as is currently thought. Further investigation into fasting and the effects of stored lipid catabolism on the pancreas in marine mammals is needed. Additional research into the zymogen status of malnourished cetaceans, both fasting and non-fasting adapted, will help discriminate changes secondary to inadequate nutritional reserves and malnutrition. This may reveal more conclusive results; however, we have not ruled out zymogen as a potentially important indicator of nutritional stress in whales, and it should be monitored as such until additional supporting data can be obtained.

Terminal airway fibromuscular hyperplasia was related to both age and cadmium concentration (renal and hepatic) in the bowhead whale. As was found in the kidney, pulmonary fibromuscular hyperplasia and age were positively correlated; however, in lung tissue the increase was non-linear. Age-related increases in pulmonary collagen content have been noted in laboratory rodents (Takubo *et al.*, 1999). Juvenile bowhead whales were found to have very little to no fibromuscular hyperplasia present, but subadults showed a rapid increase in fibromuscular hyperplasia, which then stabilized at a moderate level. Adults maintained this moderate level of change with occasional occurrence of severe fibrosis noted in older whales. These individuals were found to have increased collagen deposition at other sites (lung, blubber) as well (Rosa, unpublished data). We explored the possible relationship between hepatic and renal Cd concentrations and pulmonary fibromuscular hyperplasia. Cadmium has been related to pulmonary fibrosis in laboratory and domestic animals (Morgan *et al.*, 1997; Stoev *et al.*, 2003), though the route of exposure is often inhalation. We found that renal and hepatic Cd concentrations increased linearly with age, however, this pattern did not correspond with the initial increase and subsequent plateau in pulmonary fibromuscular hyperplasia that was observed in whales starting in late juvenile/early subadult periods. There was no evidence that the fibromuscular hyperplasia impeded air exchange. We suspect these changes to be developmental, with fibromuscular hyperplasia in the terminal airways directly related to an increased frequency of diving and increased athletic diving ability. Bowhead whales are not deep divers (Lowry,

1993); however, recently weaned calves must obtain their own food, necessitating increased diving activity during the time that the initial, mild fibromuscular hyperplasia is seen, and all whales older than juveniles were found to have some degree of pulmonary fibromuscular hyperplasia.

Brown granular pigment, identified as hemosiderin, was prominent in many of the livers. Iron was distributed predominantly within the hepatocytes and was, in some cases, distributed according to a decreasing peri-portal to centrilobular gradient. This distribution is consistent with patterns seen in human iron hyperabsorption syndromes (Turlin and Deugnier, 1998), including hemosiderosis and hemochromatosis. There was minimal mesenchymal distribution noted with little iron deposited within the sinusoidal cells that could be identified (Kupfer, endothelial and stellate/Ito cells). Dietary history (ingestion of prey high in Fe) and histologic findings suggest a condition of iron hyperabsorption in the bowhead whale, which may be a normal physiologic adaptation for this species. Bowhead whale blood and muscle are rich in hemoglobin and myoglobin, respectively, and both depend upon iron for proper function. No lesions accompanied the pigment accumulation and there was no seasonal, gender or age association noted with degree of hemosiderosis. Hemosiderosis has been sporadically noted in grey and beluga whales, as well (Dailey *et al.,* 2000; Woshner *et al*, 2002). In the bowhead whale, this may be an adaptive response to seasonal feeding. The sporadic distribution of iron in liver tissue may be related to feeding. Amount of prey ingested, type of prey ingested and the location of foraging, and thus iron exposure, are variable throughout the bowhead whale migratory pathway (Lowry, 1993). The relationship between splenic EMH and pigment accumulation should be explored further, as these findings may relate to mysticete hematopoiesis. A more complete and well-validated approach to pigment accumulation in marine mammal tissues is recommended.

We report an unexpectedly high prevalence of splenic EMH, hepatic lipidosis, renal interstitial fibrosis and pulmonary fibromuscular hyperplasia in relatively normal, healthy bowhead whales from the BCBS stock. Although there is considerable variability in the occurrence and severity of these unusual microanatomic features, none of these findings appear to cause health problems. The occurrence of similar findings in other mysticete or cetacean species, in general, is unknown, and will remain as such until opportunities to collect, properly preserve and analyze tissues samples arise. There are many research

questions that remain to be answered, and formalinized tissues are needed to perform these investigations. The recognition of species-specific histology, which normally would be considered as pathological change in more commonly reviewed species (i.e. terrestrial mammals), is critical to this research effort. Histological tissue collection protocols should be established for use during the rare opportunities that arise to obtain fresh tissues from healthy mysticetes. These protocols should be considered a routine and important part of health assessment efforts.

6.5 Acknowledgements

We express our appreciation to the Department of Wildlife Management, North Slope Borough (Barrow, Alaska), the Cooperative Institute for Arctic Research (CIFAR), UAF INBRE (Idea Networks for Biomedical Research Excellence) and NOAA for generous funding and logistical support. We also thank the Inupiaq subsistence whalers in Barrow and Kaktovik and the Alaska Eskimo Whaling Commission (AEWC) for graciously allowing us to access these samples. Individual thanks to Gay Sheffield, Victoria Woshner, Lara Dehn, Mike Castellini, Doug DeMaster, Cyd Hans, Craig George, Benny Akootchook and Chris Terzi, for their help with the manuscript, tissue collection and analytical support. Bowhead whale samples were collected under the authority of permit #932-1489-00 for the MMHSRP program (Dr. Teri Rowles). Support for this project came from CIFAR and Alaska INBRE grant number 2 P20 RR016466 from the National Center for Research Resources, National Institutes of Health, Dept. of Health and Human Services.

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Table 6.1. Key to tissues graded and analyzed in the bowhead whale during the Inuit subsistence hunt in Northern Alaska between 1998 and 2002. Autolyzed tissues are not included in the sample sizes listed below.*

Tissue collected	n
Spleen	45
Kidney	63
Lung	40
Pancreas	28
Liver	59

* Other tissues collected, but not included in this analysis include: brain, eye, tongue, trachea, adrenal gland, spinal cord, GI tract (forestomach, stomach, duodenum, jejunum, ileum, colon), blubber, epidermis, pituitary gland, lymph nodes (from several regions), muscle, heart, bladder, testes, ovaries, uterus.

Table 6.2. Guidelines used during the histological scoring of bowhead whale tissues (spleen, kidney, lung, pancreas and liver).

Spleen

Extramedullary hematopoeisis (EMH) was assessed in the spleen. Each slide was interpreted and given 0, 1 or a 2 designation:

0	No EMH present
1	Mild EMH present (1-2 areas of EMH per 40x field)
2	Marked EMH present (> 2 areas of EMH per per 40 x field)

Kidney

Fibrosis

4	No fibrosis or muscular hyperplasia present
5	Mild fibrosis (glomerular wall measurements averages 3-10 um, collagen loosely arranged around glomeruli)
6	Moderate fibrosis (glomerular wall measurements averages 10-20 um, collagen loosely arranged around glomeruli
7	Severe fibrosis (glomerular wall measurements averages >20 um, collagen densely arranged around glomeruli)

Lung

Fibrosis

4	No fibrosis or muscular hyperplasia present
5	Mild fibrosis (1-10 um terminal alveolar width), minimal muscular hyperplasia
6	Moderate fibromuscular hyperplasia (1-20 um terminal alveolar width)
7	Severe fibromuscular hyperplasia (20-30 um terminal alveolar width)

Pancreas

0	< 25% of cytoplasm occupied by zymogen granules: Zymogen depleted
1	> 25% of cytoplasm occupied by zymogen granules: Zymogen adequate

Liver

Table 6.2. continued

Lipidosis	
0	No evidence of hepatic lipidosis
1	Mild hepatic lipidosis (>1 adipocyte per 80x field)
2	Marked lipidosis
Pigment	
0	No pigment
1	Mild pigment accumulation (< 25% affected cells per 40x field)
2	Marked pigment accumulation (>25% of cells per 40x field)
Fibrosis	
0	No fibrosis
1	Mild periportal fibrosis (_ to _ um in thickness)
2	Severe periportal fibrosis (> _ um in thickness)

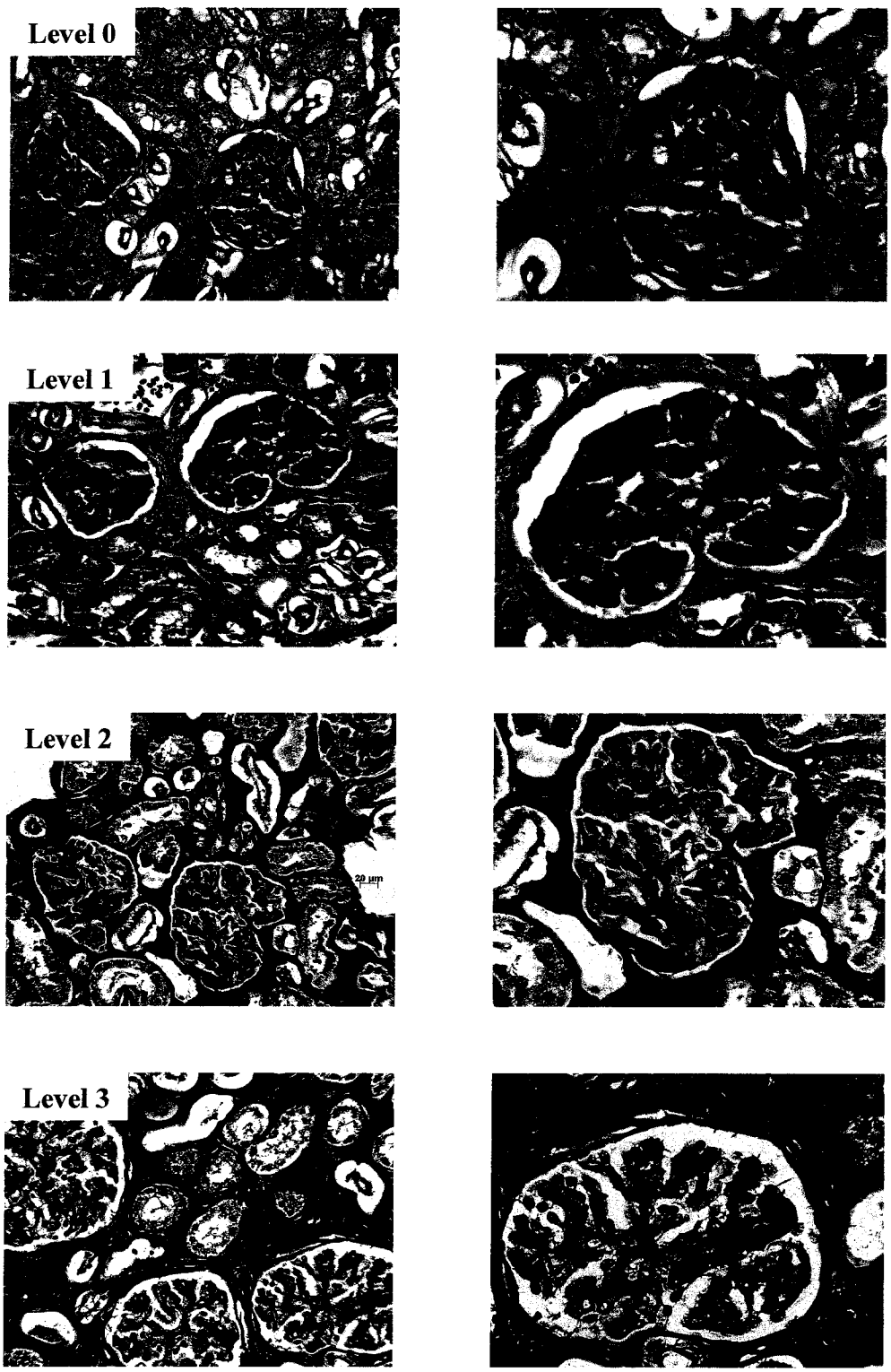


Figure 6.1. Levels 0-3 of kidney fibrosis observed in the bowhead whale. Magnification 200x on left column, 400x in right column.

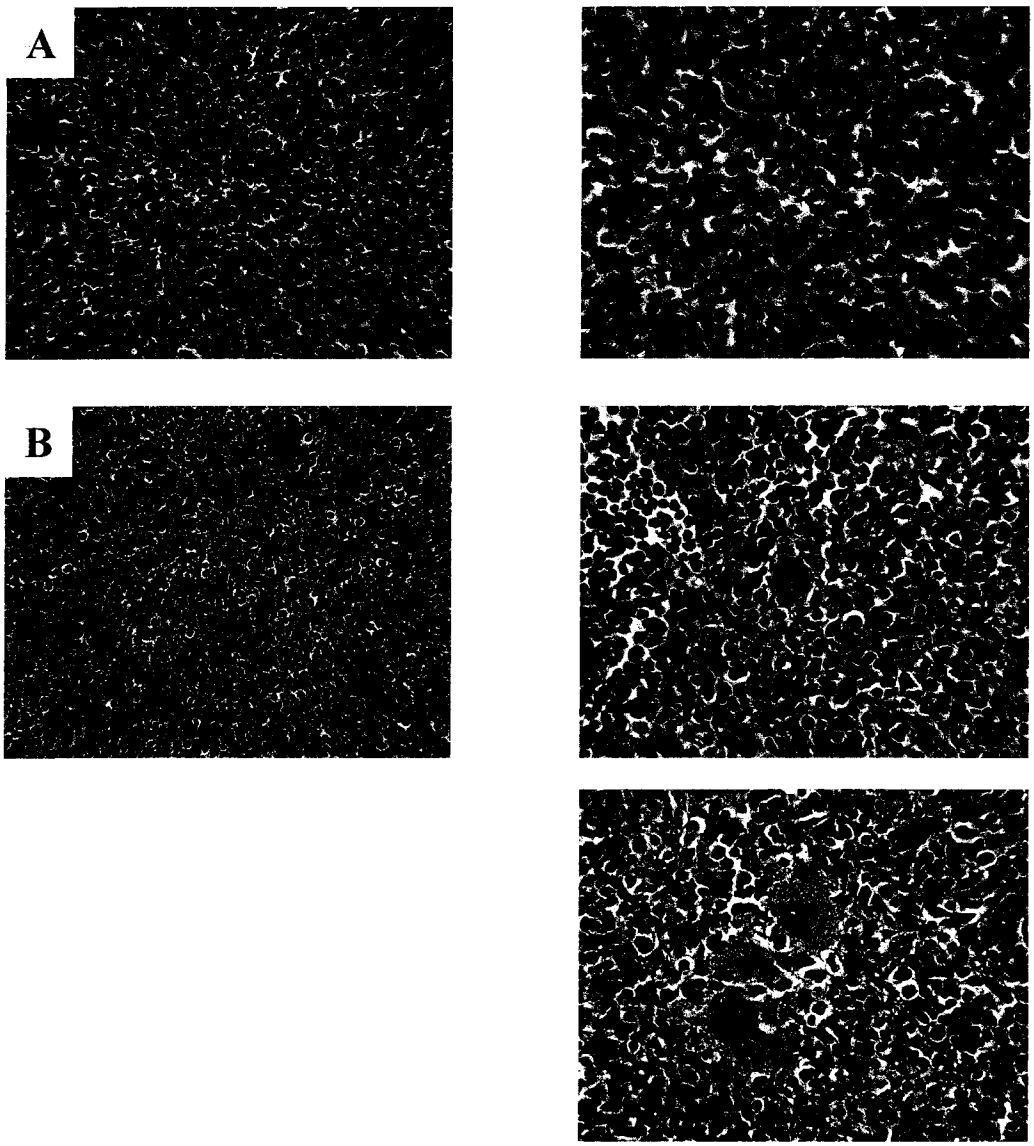


Figure 6.2. Normal spleen (A) and spleen with EMH (B) observed in the bowhead whale. Magnification 200x on left column, 400x in right column.

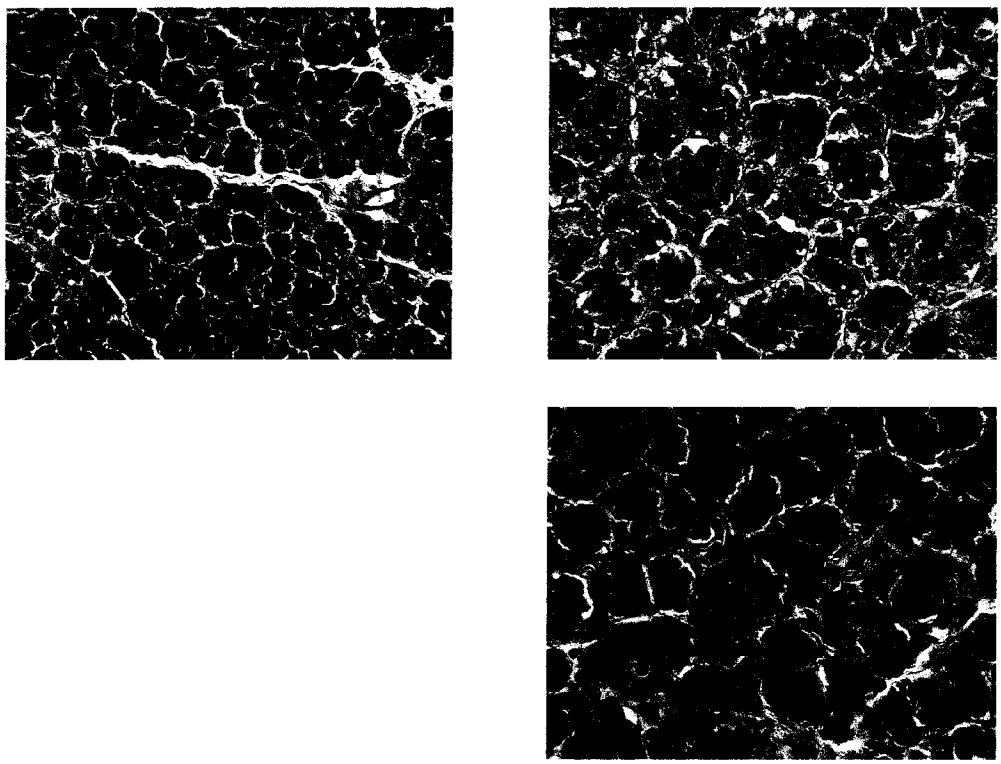


Figure 6.3. Normal pancreas with prominent zymogen granules observed in the bowhead whale. Magnification 200x on left column, 400x in right column.

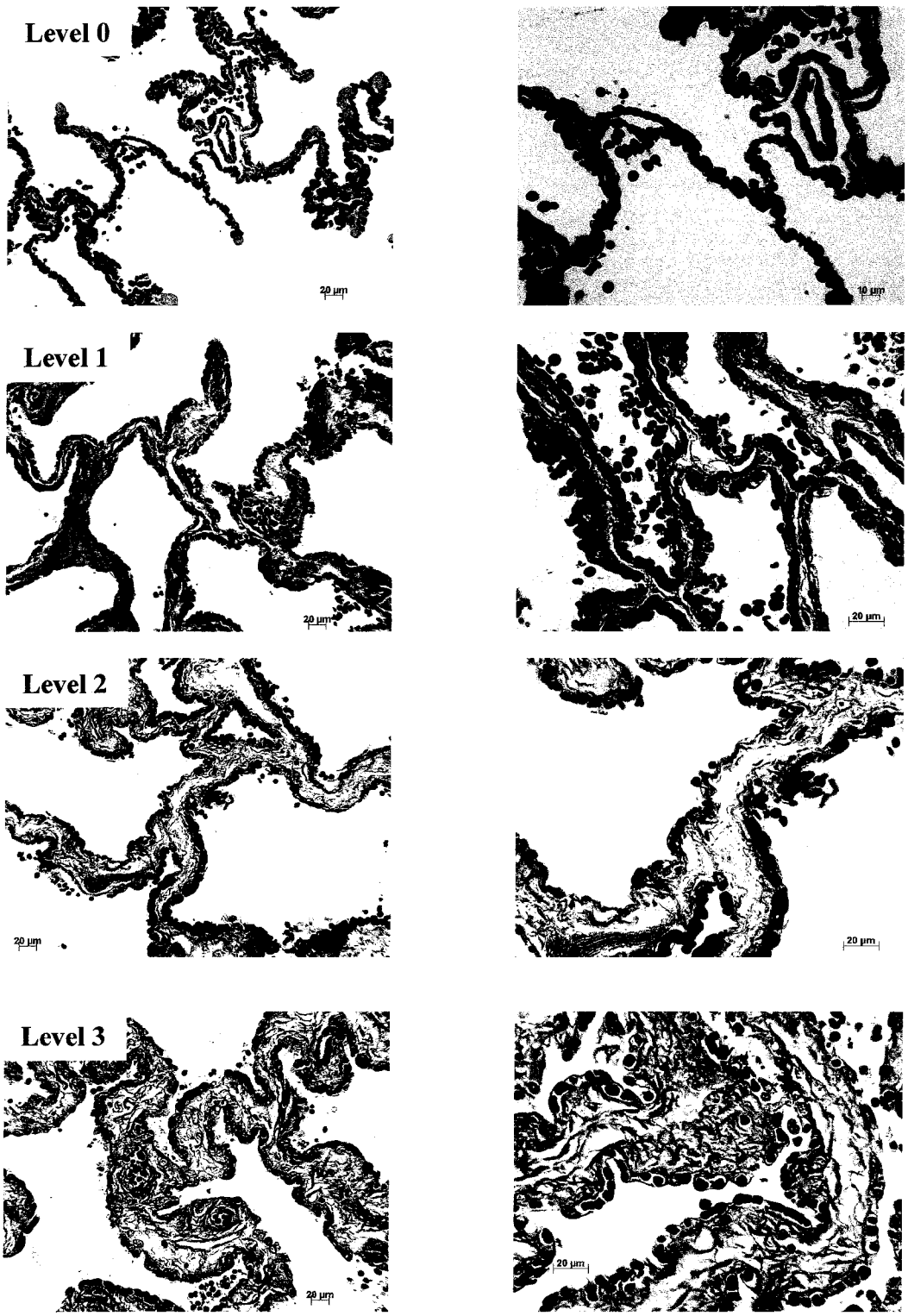


Figure 6.4. Levels 0-3 of pulmonary fibromuscular hyperplasia observed in the bowhead whale. Magnification 200x on left column, 400x in right column.

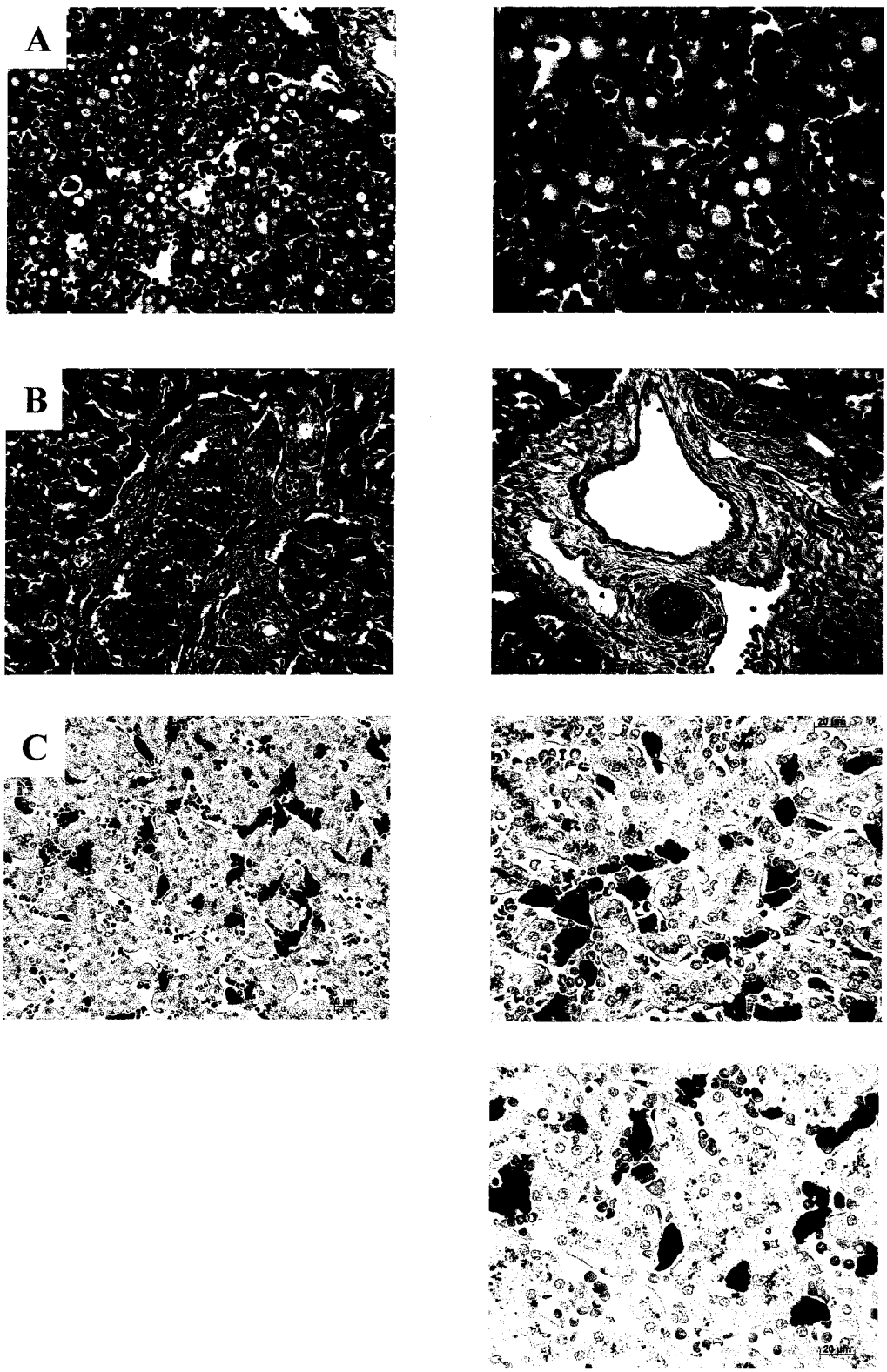
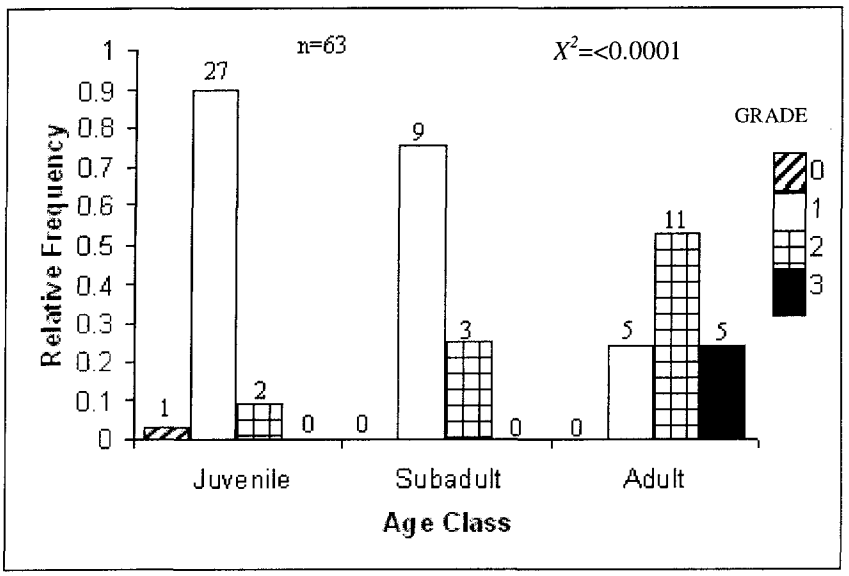
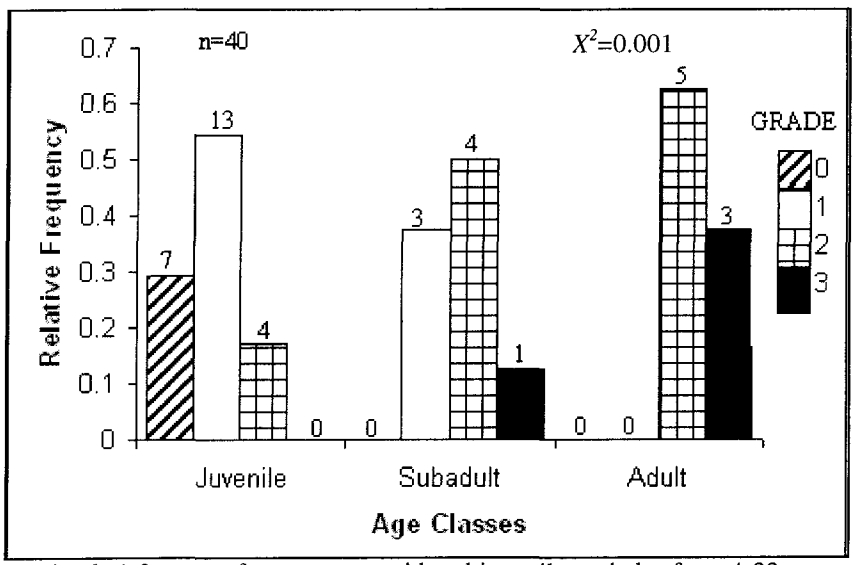


Figure 6.5. Histological findings in liver tissue of the bowhead whale. Liver with hemosiderin and lipidosis present (A, hematoxylin and eosin stain), periportal fibrosis (B, hematoxylin and eosin stain) and hemosiderin (C, Prussian blue stain) observed. Magnification 200x on left column, 400x in right column.

A. Kidney: Renal fibrosis vs. age class



B. Lung: Pulmonary fibromuscular hyperplasia vs. age class



*Animals 1-3 years of age were considered juveniles, whales from 4-22 years of age in males and 4-25 years of age in females were considered subadults and whales >22 years of age (males) and >25 years of age (females) were considered adults.

Figure 6.6. Relative frequency bar charts for significantly different results for renal fibrosis and pulmonary fibromuscular hyperplasia in the bowhead whale. Each bar chart represents the proportion of whales from each histological grade present in each age class.*

A. B.

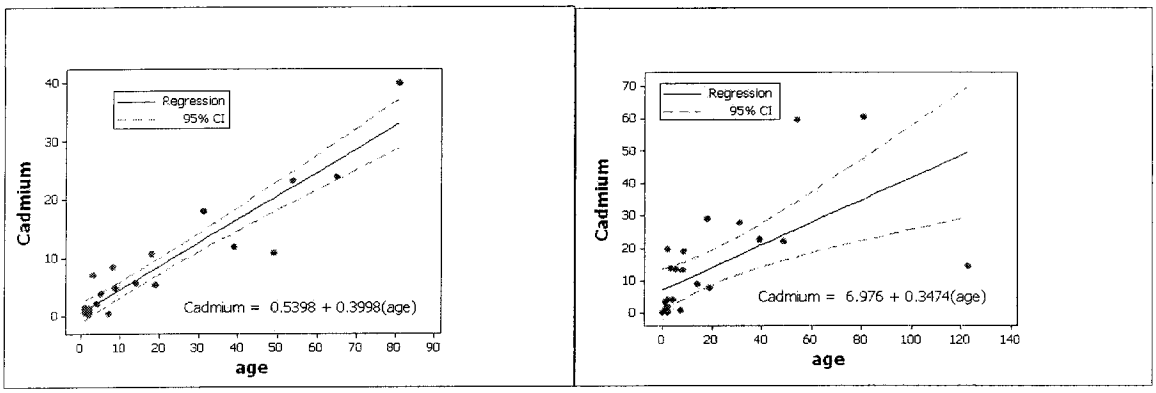


Figure 6.7. Concentrations of essential and non-essential elements in (a) liver and (b) kidney tissue (μg/g wet weight) from the bowhead whale (Balaena mysticetus) plotted against age (years). n=26.

Chapter Seven

Dermal collagen distribution and its relationship to lipid and blubber thickness in the bowhead whale (*Balaena mysticetus*).[6]

7.0 Abstract

Blubber is integral to survival of marine mammals, including large whales, which employ it for a variety of uses. Bowhead whales have among the most extensive blubber found in cetaceans. We described varying blubber thickness at six different anatomic sites on the bowhead whale (n=49 whales). Pregnant females had the thickest blubber, compared to non-pregnant females and male adults as averaged across all sampling sites on the whale. The distribution of collagen and lipid at these sites was investigated along with analysis of collagen and lipid at several depths (strata) per site. Blubber collagen percentage (BCP) did not differ significantly between spring and fall seasons, nor did it differ significantly among sites on the whale. The innermost (closest to muscle) of the five blubber layers analyzed contained a significantly higher BCP than the outer depths. However, we found no difference in BCP found among the outer four depths. Both gender group and age were found to affect BCP. Non-pregnant and pregnant adult females were statistically indistinguishable; however, both of these groups contained a significantly higher percentage of collagen in their blubber than adult males. A positive linear relationship was found between age and BCP. There was no correlation between lipid content and BCP or between lipid content and blubber thickness. However, we noted a positive relationship between blubber thickness and BCP. Principle components analysis revealed a positive relationship between blubber thickness and BCP with a marked spatial separation between these two variables and percent lipid in blubber. The implications of these findings are discussed with respect to the collection of morphometric data and marine mammal health assessment.

Keywords: Arctic, Bowhead whale, disease, histology, monitoring, morphometrics, sampling strategy

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[6] Rosa, C., O'Hara, T.M., Gray, M.J.,Ylitalo, G.M., Blake, J.E. Dermal collagen distribution and its relationship to lipid and blubber thickness in the bowhead whale (*Balaena mysticetus*).
Prepared for submission to the Journal of Cetacean Research and Management

7.1 Introduction

Recent declines in several marine mammal populations have stimulated interest in developing reliable and straightforward methods to assess the health of individuals and populations of marine mammals (Barron *et al.*, 2003; Rolland *et. al.*, 2005; Burek *et al.*, 2005). Marine mammal blubber and the overlying epidermis are, in general, abundant and relatively easy to sample. It has therefore been the organ of choice for many studies assessing genetics, body condition, nutritional status, feeding ecology, and concentrations of contaminants (Krahn *et al.*, 1997; Koopman *et al.*, 2002; Hobbs *et al.*, 2003; Dalebout *et al.*, 2005).

Blubber is adipose tissue with a support network composed primarily of collagen and, depending upon species, elastin (Iverson, 2002, Toedt *et al.*, 1997). Bowhead whales invest heavily in developing and maintaining the thick blubber layer that comprises up to 50% of their total body mass (J.C. George, personal communication). Blubber thickness is relatively simple to measure in cetaceans, and has been used in the past as a surrogate measure of body condition (Slipjer, 1954; Ash, 1956). However, recent research has shown that the quantity of blubber, particularly as measured by thickness, may not accurately assess its quality (i.e., amount of lipid, essential elements and vitamins present) and therefore is unreliable as a measurement of health status or body condition (Evans et al., 2003; Mau, 2004; Rosa 2005, unpublished data). Blubber serves as a sizable reserve for many of the vitamins and minerals that are essential for health. Fatty acids present in this tissue are essential for body function (Wetzel and Reynolds, 2004). Measuring the amount and types of lipid present is important to properly evaluate the quality of blubber (Lockyer *et al.*, 1985; Koopman *et al.*,1996; Evans *et al.*, 2003; Mau, 2004). This may be especially true as it relates to the assessment of body condition and health evaluation.

There are a number of published studies on the lipid content of blubber (Lockyer *et al.*, 1984; Lockyer *et al.*, 1985; Aguilar and Borrel, 1990); however, there is little detailed information available on the proteinaceous framework of blubber, which is comprised largely of collagen (Orton and Brodie, 1985; Toedt *et al.*, 1997; Hamilton *et al.*, 2004). This component can be quite extensive in larger cetaceans and remains after lipid depletion in malnourished marine mammals (Koopman *et al.*, 2002). It is also slow to degrade after death (i.e. stranded animals) (Pierce *et al.*, 2004) which is not the case for lipids (Krahn *et al.*,

2001; Mau, 2004). These factors illustrate its potential use as a measure of body condition in marine mammals of variable body conditions, both pre- and post-mortem.

"Blubber" is from the Middle English word *bluber,* which means to bubble or foam. This word was used as a colloquialism by whalers to describe the thick layer of fat between the epidermis and the muscle layers of whales and other marine mammals (Merriam-Webster, 2004). It was later adopted by scientists for the same purpose; however, specific morphologic and histologic definitions were never designated. In the historical scientific literature, there are three main definitions: Parry (1949: p. 13) stated that 'Blubber comprises the epidermis, dermis and hypodermal tissues'. Parry's definition includes all tissue external to the muscle fascia. Slipjer (1962: p. 43) indicated that the dermis is a thin, dense layer below the epidermis that contains no fat. He considered the material between this thin dermis and the internal muscle layer to be hypodermis, which is analogous to the panniculus adiposis in terrestrial mammals and refered to this as blubber. Haldiman and Tarpley (1993: p. 78) defined blubber as fat in the collagenous matrix below the epidermis forming a 'modified layer of reticular dermis'. They refered to the hypodermis, a layer distinct from the reticular dermis/blubber, as the innermost layer of loose fat/collagenous tissue with muscle fibers (Haldiman *et al,* 1981; 1985; Haldiman and Tarpley, 1993). This is the definition that we have adopted for this research.

The disagreement about the precise definition of blubber can be problematic for sample collection and research comparisons. It is important to define, because research has shown stratification of various components in the adipose tissue surrounding marine mammal bodies (Aguilar and Borrel, 1990; Koopman *et al.*, 2002; Best *et al.*, 2003; Krahn *et al.*, 2004). Additionally, different blubber layers may perform alternative functions and accumulate and deplete lipid and protein at variable rates. In the harbor porpoise, the most external adipose tissue housed within a collagen framework is thought to be structural and insulatory, with the internal, more loosely arranged and highly vascular adipose tissue thought to serve as an energy store (Koopman *et al.*, 2002). Regardless of definition, blubber is composed of lipid, water and protein, the latter being comprised largely of collagen which is known to change with age in mammals (Resier *et al.*, 1987; Yamauchi *et al.*, 1988; Reiser, 1994).

The bowhead whale, *Balaena mysticetus*, inhabits a cold and often ice-dominated environment. Many evolutionary adaptations have resulted in a body form adept at handling extreme environmental conditions. The development of a thick, adipose layer ("blubber") that covers the entire body functions as an energy source, a support structure, a source of insulation and buoyancy, and in protection and thermoregulation. (Iverson, 2002; Ryg *et al.*, 1988) Known for its remarkable longevity (George *et al.*, 1999, Rosa *et al.*, 2004, Lubetkin *et al.*, 2004), the bowhead whale may live over 150 years. These distinct characteristics make an understanding of the changes in collagen that occur over age, from season to season and between genders necessary to properly interpret blubber data and health-related findings.

A morphological and histological assessment of blubber obtained from different anatomic sites on the bowhead whale was conducted as part of a bowhead whale health assessment study. Research focused on the thickness and collagen composition of bowhead whale blubber, with a lesser emphasis on lipid composition, which was covered in detail by Mau (2004). Blubber morphology was evaluated by season, gender, reproductive status, and age. Blubber collagen percentage was compared at 6 core sites at 5 depths each on the whale (Figure 7.1). We examined the relationship between blubber thickness, collagen percent and percent lipid at these 30 sites and evaluated the use of BCP as a parameter for cetacean health evaluation.

7.2 Materials and Methods

7.2.1 Sample collection

Morphometric and blubber thickness measurements (Appendix 1) and full thickness blubber cores (measuring 2 cm^2 in area, from epidermis to muscle fascia) were collected from near the blowhole (approximately axillary girth) and umbilical girths of 49 bowhead whales (n=49) during the 1998-2001 spring and fall Inuit subsistence hunt in Barrow and Kaktovik, Alaska. Scientific collection was conducted with permission of the Barrow and Kaktovik Whaling Captains Association and the Alaska Eskimo Whaling Commission (AEWC) through the Department of Wildlife Management (North Slope Borough, Alaska). This permit functions under the purview of a National Oceanic and Atmospheric Administration (NOAA) permit issued to Dr. Teri Rowles (#932-1489-00 for the Marine Mammal Health and Stranding Response Program). Blubber was sampled from six sites on the whale (Figure 7.1): dorsal, lateral and

ventral using "blowhole" girth (1 meter caudal to the blowhole) and the "umbilical" girth (site demarcated by presence of umbilical scar) (Fig. 7.1). This provided blubber cores from six sites designated: BD (blowhole dorsal), BL (blowhole lateral), BV (blowhole ventral), UD (umbilicus dorsal), UL (umbilicus lateral) and UV (umbilicus ventral). The position of the whale during butchering, extensive butchering by the hunters prior to arrival of sampling crew or dangerous ice conditions precluded collection of all 6 blubber cores for some whales. Blubber cores were divided lengthwise and one half was frozen and the other half was placed in neutral buffered 10% formalin within approximately 3-14 hours of death. Blubber core length was measured on the whale and off the whale before and after fixation in neutral buffered 10% formalin. Whales were designated male, female or pregnant/lactating female by gross findings determined during butchering.

7.2.2 Sample preparation

Following the definition developed by haldiman and Tarpley (1993), in this study, the term blubber refers exclusively to the reticular dermis and excludes the epidermis, papillary dermis, and the hypodermis. Post-fixation, the epidermis was removed at the level of the papillary dermis, and hypodermal remnants, if present, were trimmed from the samples due to the inconsistency of our ability to sample this layer from the whales. The remaining blubber core was divided into 5 equal sections (each section was equal to 20% of the core sample length, Figure 7.1). A sample was collected at a point equidistant from the top and bottom of each of these sections on a horizontal plane. These samples were processed and embedded in fresh paraffin. Tissue was cut at 5 μm thickness and four slides were made from each block. The first of the four slides was stained with hematoxylin and eosin for general histological review. The three remaining slides were subjected to three different staining protocols: Masson's trichrome, VerHoeff's elastin stain and reticulin stain (American Master*Tech Scientific, Inc., Lodi, CA). Masson's trichrome stains collagen blue and most other intracellular and extracellular protein red, VerHoeff's elastin stains elastic fibers black and the reticulin stain is a silver impregnation method that stains reticulin fibers black. Slides were stained in groups of 20, with a control slide (reindeer aorta for the trichrome and elastin stains and liver for the reticulin stain) included in each group processed for quality assurance/quality control purposes.

7.2.3 Image analysis

Prepared slides were reviewed using a Leitz Laborlux S microscope (Leitz/Leica Microsystems Inc., Bannockburn, IL). Photomicrographs and measurements were taken using a Zeiss Axiocam camera and Axiovision software (version 3.2, Carl Zeiss, Inc., Thornwood, NY). Using a labeled grid (Lovin Field Finder, Gurley Precision Instruments, Troy, New York USA), specific areas (16x magnification) were randomly selected to be digitally photographed. Photomicrographs were subsequently analyzed for blubber collagen percentage (BCP) using Image J software (Abramoff et al. 2004).

7.2.4 Lipid analysis

Blubber layers 1, 3 and 5 from the BD, BL and BV sites (Figure 7.1) were analyzed for total lipids by thin layer chromatography coupled with flame ionization detection (TLC/FID). Detailed methods are described in Krahn et. al. (2001) and Ylitalo et al. (2005).

7.2.5 Aging

A combination of baleen stable isotope analysis of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$) signatures (n=8) and/or aspartic acid racemization (AAR) of eye lens nuclei (n=38) was used to determine age in 46 of the 49 whales (George et al., 1999; Rosa et al, 2004, Lubetkin et al., 2004).

7.2.6 Statistical Analysis

Data are presented as the mean with standard deviation (SD). An analysis of variance (ANOVA) with type III sums of squares due to unequal sample sizes was used to test for seasonal, gender and age-related differences in BCP (three-way nested-factorial design with site and depth as crossed, fixed factors and landed whales nested within season). A least square means with Tukey experimentwise error rate adjustment was used for post-hoc comparison of means when the F-test for an effect was significant. A two-factor ANOVA was used to determine differences in blubber thickness in pre and post-fixation samples between sites. ANCOVA was used to test for sex effect with age as a covariate (collagen) and for differences between sex classes and season cohorts while partitioning variation associated with age (blubber thickness) with type III error analysis. A simple linear regression was used to model collagen versus age and blubber thickness versus age. Principle components analysis was employed to evaluate the relationship between BCP, percent lipid and blubber thickness (cm). Additional univariate analyses were

performed in order to explore the relationship between lipid and collagen. All statistical analyses were performed on the SAS operating system (SAS Institute, Inc., Cary, NC). Values were considered significantly different at $P \leq 0.05$.

7.3 Results

7.3.1 Gross anatomy and histology

Collected core samples consisted of epidermis, papillary dermis, reticular dermis and hypodermis (Figure 7.2a). The epidermis was highly pigmented and parakeratotic. The papillary dermis consisted of a band of dense collagen, approximately 0.3 cm in width, which formed interdigitations with the epidermal tissue (dermal papillae). The reticular dermis consisted of adipose tissue distributed in a structural network of collagen fibers and bundles. The hypodermis lacked this internal support system, was variable in thickness and often had fine skeletal muscle fibers running within it (Figure 7.2b). The soft, amorphous fat that comprised the hypodermis sometimes remained on the whale secondary to sampling technique. It was not always present as part of the core sample and was not included in the histological analyses or in the off-whale core measurements, as it was very difficult to measure post-removal due to its amorphous characteristics (i.e., it tends to stretch and tear upon removal from the whale). Twenty-nine of the 49 bowhead whales examined, predominantly "fat" juvenile whales, exhibited a hypodermal layer beneath the reticular dermis, measuring between 1 and 9 cm thick. This layer contained little to no collagenous support structure. The dermis was separated from the hypodermis by a thick layer of collagen in many cases (Figure 7.2a).

There was a small, statistically significant difference found between pre and post-fixation measurements of blubber cores (0.84 cm, P=0.002). Pre-fixation core length measurements were always greater than post-fixation measurements. Blubber site interaction with treatment (pre vs. post measurements) was mildly significant (F=3.93, P=0.053).

Selective histological staining showed that the connective tissue matrix in bowhead whale blubber consisted almost exclusively of collagen, with elastin and reticulin being present only in association with

vasculature (Figure 7.3a). The hypodermal layer consisted mainly of adipocytes with a small amount of fine collagen irregularly distributed within (Figure 7.3b).

7.3.2 Blubber collagen percent (BCP)

BCP did not differ significantly between spring (n= 13, x= 13.22, SD= 3.94) and fall (n=13, x=10.62, SD=3.94), nor did it differ significantly among sites on the whale (Table 7.1). There was a difference in BCP among the five depths; depth five contained a significantly higher percentage of collagen than depths 1 through 4 (Table 7.2, F=13.92, P <0.0001). Differences among depths 1 through 4 were not significant (F=1.68, P>0.2071), as confirmed with post-hoc testing (least square means with Tukey experimentwise error rate adjustment, Table 7.2).

Gender (F= 7.44, P= 0.0007) and age (F=3.44, P=<0.0001) affected BCP. When all sites/depths of blubber were considered, the BCPs of non-pregnant adult females (n=238, x=11.53, SD=9.42) and pregnant adult females (n=93, x=15.08, SD= 9.93) were statistically indistinguishable; however, both of these groups contained a significantly higher BCP than adult males (n=170, x=10.65, SD=9.07). When site BD alone was compared among gender and reproductive groups, only the pregnant females (n=4, x=15.52, SD= 2.10) were statistically distinguishable, with non-pregnant adult females (n=13, x=10.95, SD=3.94) and adult males (n=9, x=11.72, SD=4.36) being lower in BCP. Five pregnant females and one lactating female composed the "pregnant" group (we did not have BCP information for all pregnant whales measured). The lactating female has been included with the pregnant females as fresh wounds in her genital region indicated that she was periparturient.

A linear relationship was detected between age and average BCP (F=13.39, P= 0.001, Figure 7.4). Thirty-seven percent of the variation in collagen could be explained by the age of the whale (R^2=0.37).

7.3.3 Blubber thickness

Blubber thickness varied significantly among sampling sites (Table 7.3). Significant thickness relationships are as follows: BD blubber cores were significantly thicker than BL, BV, UL cores. UV blubber cores were significantly thicker than BL cores.

Pregnant females had the thickest blubber (n=6, x=27.31, SD=2.12), compared to non-pregnant females (n=27, x=21.03, SD=3.48) and males (n=16, x=21.76, SD=3.14) as averaged across all sampling

sites. Age had a significant (P=0.05) effect on blubber thickness (Figure 7.5). Thirty-eight percent of the variation in blubber thickness could be explained by age (R^2=0.38). Univariate Pearson correlations showed no correlation between percent lipid and blubber thickness (R=0.127, P=0.57) and no correlation between percent lipid and BCP (R=-0.123, P=0.57); however, there was a correlation noted between blubber thickness and BCP (R=0.420, P=0.05).

7.3.4 Principle components analysis

Principle components analysis indicated a relationship between blubber thickness and collagen % with a marked spatial separation between these two variables and lipid % (Figure 7.6). Percent total lipid data for sites BD, BL and BV are summarized in Table 7.4. There was no correlation between lipid and collagen (R^2=-0.012, P=0.59) or lipid and blubber thickness (R^2=0.13, P=0.57) via univariate Pearson correlation analysis, however this analysis did indicate a significant positive relationship between blubber thickness and BCP (R^2=0.42, P=0.05).

7.4 Discussion

The cross linking structural matrix responsible for much of the resiliency of the bowhead whales' substantial blubber layer is exclusively collagen. Contrary to what has been reported for odontocetes (e.g., Toedt *et al.*, 1997; Hamilton *et al.*, 2004), this structural support system for the adipose tissue in bowhead whale blubber contains no histologically detectable elastin or reticulin fibers. The reinforcement and spring provided by the elastin fibers in relatively rapid swimming toothed whales may be important for odontocete locomotion but unnecessary to meet the demands of bowhead whale locomotion, with insulation and energy storage for the fasting period being blubber (lipid) functions of greater importance in this species. Locomotion differs substantially between small to medium odontocetes and large mysticetes, with the bowhead whale reaching average swimming speeds of ~3 km/h (~10% of the speed of a typical porpoise) (Orton and Brodie, 1985; Zeh *et al.*, 1993). Additional characterization of blubber stucture in other cetacean species would assist in investigations of its relationship to locomotion and streamlining in mysticetes versus odontocetes and help to document species-specific differences present in its structural framework.

Lipid stratification in the blubber of several cetacean species has been reported (e.g., Lockyer *et al.*, 1984; Lockyer *et al.*, 1985; Aguilar and Borrel, 1990). In several mysticete species, the outer layer of blubber is the most stable, forming a long-term depot for lipid. The middle layer often contains the highest lipid content. The innermost layer has been found to be the most variable and dynamic in composition, and is thought to reflect the nutritional status of the animal (Aguilar and Borrel, 1990, Ackman *et al.*, 1965, 1975a, 1975b, Mau, 2004). Blubber is also stratified anatomically. Ackman *et al.* (1975b) noted that Heyerdalh (1932) described a unique layer of fatty tissue (called "isterlag") interposed between two layers of connective tissue in the innermost portion of the blubber of "fat" fin whales and sei whales. Ackman *et al.* (1975b) described a similar tissue in sei whales and noted that its occurrence was not restricted to "fat" whales, but seemed to be widespread in all whales examined. Lockyer (1987) found this intermediate layer to be present inconsistently in Icelandic fin whales in comparison to the Antarctic population of fin whales studied and hypothesized that the layer might be secondary to the greater lipid reserves needed for survival in the Antarctic whales (Lockyer *et al.*, 1987). Bowhead whales, especially "fat" recently weaned juveniles, exhibit this "isterlag" layer, which we propose corresponds to hypodermis (Figure 7.2a), with the thick, structured blubber layer external to this representing a specialized form of the reticular dermis (Haldiman and Tarpley, 1993). In Barrow, Alaska, local residents refer to this as "double maktak" (J.C. George, personal communication). This innermost layer of fat distributed beneath the reticular dermis was present on 25 of the 49 samples (1 to 9.5 cm in depth). This layer has little to no collagenous supporting matrix and is morphologically unlike that which is found in the reticular dermis. This lack of structure proved problematic for depth measurements of hypodermis pre- and post-removal from the whale. Measurement of hypodermal thickness depended heavily upon the way the whale was positioned on the ground, as this amorphous layer easily expanded or compressed under the weight of the whale or from the tension placed on this tissue. When the full thickness cores were removed from the whale, the hypodermal layer lost its *in situ* shape and was difficult to measure accurately. Investigations into ultrasonic differences between the characteristics of the reticular dermis and the hypodermis would be valuable, as measuring this layer while the subject is still floating (neutrally buoyant) is likely to be the most accurate way to assess its depth. The increased BCP in the innermost layer of blubber (BD5) most likely conforms to the collagenous

layer that compresses the true hypodermis or "isterlag" layer between itself (the innermost layer of the reticular dermis) and the muscular layer that underlies the hypodermis. We speculate that this layer is present in all cetaceans, exhibits species-dependent development and is variable with age- and season-dependent fattening. Histological and embryological research is required for further clarification of this structure and a more definitive assessment. Understanding the anatomy of blubber, especially the hypodermis which is innermost and potentially most dynamic and metabolically active region, is important for interpretation of lipid physiology research. This layer is likely to be critical to the interpretation of recent feeding activity and health status in general.

Blubber is not distributed homogeneously over the body of the bowhead whale. There is considerable variability present in blubber appearance and thickness along blowhole and umbilical girths with the blowhole dorsal site (BD, ~1 meter caudal to the blowhole) being the among the thickest of the six sites studied. The reason for the thick dorsal blubber region is likely to be multifactorial. The dorsal region may be the thickest to assist in the maintenance of buoyancy and upright swimming confirmation (Lockyer et al., 1984). There may also be a selective pressure present in this species for thick dorsal blubber that is effective at creating "push ups"- breathing holes made in sea ice (George *et al.*, 1989; Zeh *et al.*, 1993). Other hypotheses for this heterogeneity suggest that blubber from different regions of the body varies in relative function (i.e., locomotion, insulation or energy storage), each of which can been drawn upon preferentially, resulting in differences in blubber composition and thickness in different sites (Doidge *et al.*, 1990; Koopman *et al.* 1996). The BD site may be the most impervious to these changes. Lipid composition is also important relative to these changes. These differences and site characteristics are important with respect to tissue sampling, and may allow researchers to predetermine the most appropriate site for sampling with respect to the specific species and research question involved. Determination of specific sampling regions in free-ranging wildlife presents a logistical challenge, as a controlled environment is likely to be necessary in order to discriminate between general energetic needs and blubber catabolism for specific purposes.

Pregnant and non-pregnant adult females had significantly thicker blubber than adult males. This is similar to findings in fin whales (Lockyer et al., 1984) and is likely attributable to the large amount of

reserve energy needed by females in order to survive pregnancy and lactation and produce a viable calf. It also may involve body size, as female bowhead whales are larger than males once mature (George et al., 1999). The positive relationship found between age and blubber thickness has been reported in several other marine mammals and may relate to growth and body scaling issues (Struntz *et al.*, 2003). As mentioned previously, blubber thickness is relatively simple to use as a proxy of "health", however, the quantity of blubber present (thickness) is not the only variable affected by nutritional condition and may not be the most sensitive indicator of health or disease. The quality of the blubber present may also be affected, particularly with respect to its lipid concentration and protein/collagen content (Aguilar and Borrell, 1990). Optimally, blubber thickness data are analyzed along with lipid and protein quantification in order to assess the tissue and nutritive state of the animal more fully.

Though it was not analyzed in detail in this study, blubber lipid percentage did not influence the thickness of blubber or BCP. There was a positive relationship between blubber thickness and BCP, with these two factors grouping together, shown to be spatially separated from lipid by principle components analysis. Age is likely to be related to this grouping pattern, as it has been shown to affect both blubber thickness and BCP independently. Increased blubber thickness with aging may be mass/scale dependent, and collagen cross-linking and fiber diameter have been documented to increase with age (Lockyer et al., 1995; Resier *et al.*, 1987; Yamauchi *et al*, 1988; Reiser, 1994). The lack of association between blubber thickness and lipid content may also relate to the sample of animals represented in this study, which were mostly in robust body condition. If emaciated animals were present, a decrease in percent total lipid and/or blubber thickness would be expected.

Blubber collagen percent was affected by age; however, it did not differ significantly between spring and fall seasons, or among the six sites sampled on the whale. Among all five depths, the only variability noted was in the innermost depth of blubber (depth 5). The BCP in this layer was significantly higher than depths 1-4, which were all statistically similar. This lack of variability in depths 1-4 may be a valuable characteristic. Other blubber attributes vary by site (lipid and thickness) and depth (lipid) of sampling. The consistency of BCP values over all site and most depth locations (depths 1-4) may make a deviation from the normal range for a given age/gender group a more reliable and detectable indication of

nutritional status. However, the whales involved in this research were in good to excellent body condition and health (as defined by morphometric measurements, few gross lesions, histopathology, or other signs of disease). It is difficult to infer changes that may occur in BCP or blubber thickness in a less healthy state from these whales. Though the sample size was small (n=2), Struntz (2003) found structural fiber cross-sectional areas in emaciated bottlenose dolphins to be significantly higher than in non-emaciated animals (Struntz *et al.*, 2003). Samples from bowhead whales in poor body condition are needed to make the present research more conclusive with respect to health assessment.

Marine mammal blubber is influenced by the quality and quantity of the animal's diet, therefore, there is considerable value in using blubber thickness, lipid and BCP measurements in future health assessment projects. Data from the detailed sampling scheme employed here have shown that these measurements should be made at several sites on the whale. Our results indicate the need for additional investigations into the anatomy of blubber (gross and histological), the composition and dynamics of its framework (collagen, elastin) and the effects of different nutritional states on blubber collagen. We also emphasize the need for clear definitions of the term "blubber", especially when samples are being collected from large cetaceans that are likely to have a well-developed hypodermal layer. The use of appropriate histologic and morphologic characteristics will facilitate greater understanding of this important tissue in cetaceans.

7.5 Acknowledgements

We express our appreciation to the Department of Wildlife Management, North Slope Borough (Barrow, Alaska), the Cooperative Institute for Arctic Research (CIFAR), the Alaska IDeA Networks for Biomedical Research Excellence (INBRE) and NOAA for generous funding and logistical support. We also thank the Inuit subsistence whalers in Barrow and Kaktovik and the Alaska Eskimo Whaling Commission (AEWC) for graciously allowing us to access these samples. Individual thanks to Gay Sheffield, Victoria Woshner, Lara Dehn, Mike Castellini, Doug DeMaster, Cyd Hans, Craig George, Benny Akootchook and Chris Terzi for their help with the manuscript, tissue collection and analytical support. Bowhead whale samples were collected under the authority of permit #932-1489-00 for the MMHSRP program (Dr. Teri Rowles). Support for this project came from CIFAR and Alaska INBRE grant number 2 P20 RR016466 from the National Center for Research Resources, National Institutes of Health, Dept. of Health and Human Services.

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Table 7.1. Blubber collagen percentage (BCP) in the bowhead whale collected from six anatomic sampling sites[1].

Variable		n	$\bar{x}$	SD
Site				
	BD	23	11.33	4.66
	BL	17	13.31	5.14
	BV	19	10.89	4.35
	UD	19	10.23	3.81
	UL	15	11.95	1.73
	UV	13	14.89	5.47

[1] BD (blowhole dorsal), BL (blowhole lateral), BV (blowhole ventral), UD (umbilicus dorsal), UL (umbilicus lateral) and UV (umbilicus ventral). The blowhole girth is ~ 1 meter caudal to the blowhole.

Table 7.2. Blubber collagen percentage (BCP) values per depth in the bowhead whale as averaged over all six anatomic sampling sites (BD, BL, BV, UD, UL, UV).

Variable	Level	n	$\bar{x}$ [1]	SD
Depth[2]	1	24	12.14^{A}	3.51
	2	25	7.88^{A}	2.54
	3	25	7.47^{A}	2.26
	4	25	10.66^{A}	4.68
	5	25	20.77^{B}	11.53

[1] Means within variables with different letters are significantly different ($P < 0.0009$) by Tukey-Kramer adjustment for multiple comparisons
[2] Each of the five depths is 20% of the total core length, with depth one being the most external site and depth 5 being the most internal site.

Table 7.3. Blubber thickness (cm) in the bowhead whale collected from six anatomic sampling sites[1].

Variable		n	$\bar{x}^2$	SD
Site	BD	44	23.95*	6.11
	BL	35	20.77	5.20
	BV	41	20.93	3.86
	UD	41	22.60	4.43
	UL	36	21.03	3.29
	UV	37	22.98†	4.02

[1]BD (blowhole dorsal), BL (blowhole lateral), BV (blowhole ventral), UD (umbilicus dorsal), UL (umbilicus lateral) and UV (umbilicus ventral). The blowhole girth is ~ 1 meter caudal to the blowhole.
[2] Means within variables are significantly different ($P < 0.0393$) by Tukey-Kramer adjustment for multiple comparisons for * (significantly thicker than BL, BV, and UL sites) and † (significantly thicker than BL).

Table 7.4. Percent total lipid from blubber cores collected on the blowhole girth[1] (mean) and at three anatomic sites from the bowhead whale (*Balaena mysticetus*).

Variable				n	$\bar{x}$	SD	Range
Over all sites on the blowhole girth:							
		Depth	1	51	53.33	12.58	(27-75%)
		Depth	3	51	60.57	13.68	(32-85%)
		Depth	5	51	55.18	14.50	(23-85%)
Site	BD	Depth	1	19	49.92	13.94	(28-72%)
		Depth	3	20	58.90	12.04	(44-83%)
		Depth	5	19	54.74	13.09	(34-74%)
	BL	Depth	1	12	48.83	11.67	(30-69%)
		Depth	3	12	59.42	12.68	(40-80%)
		Depth	5	13	57.85	13.74	(38-85%)
	BV	Depth	1	16	52.75	13.28	(27-74%)
		Depth	3	17	57.00	14.40	(32-85%)
		Depth	5	15	56.53	13.71	(39-82%)

[1]BD (blowhole dorsal), BL (blowhole lateral), BV (blowhole ventral). The blowhole girth is ~ 1 meter caudal to the blowhole.

A.

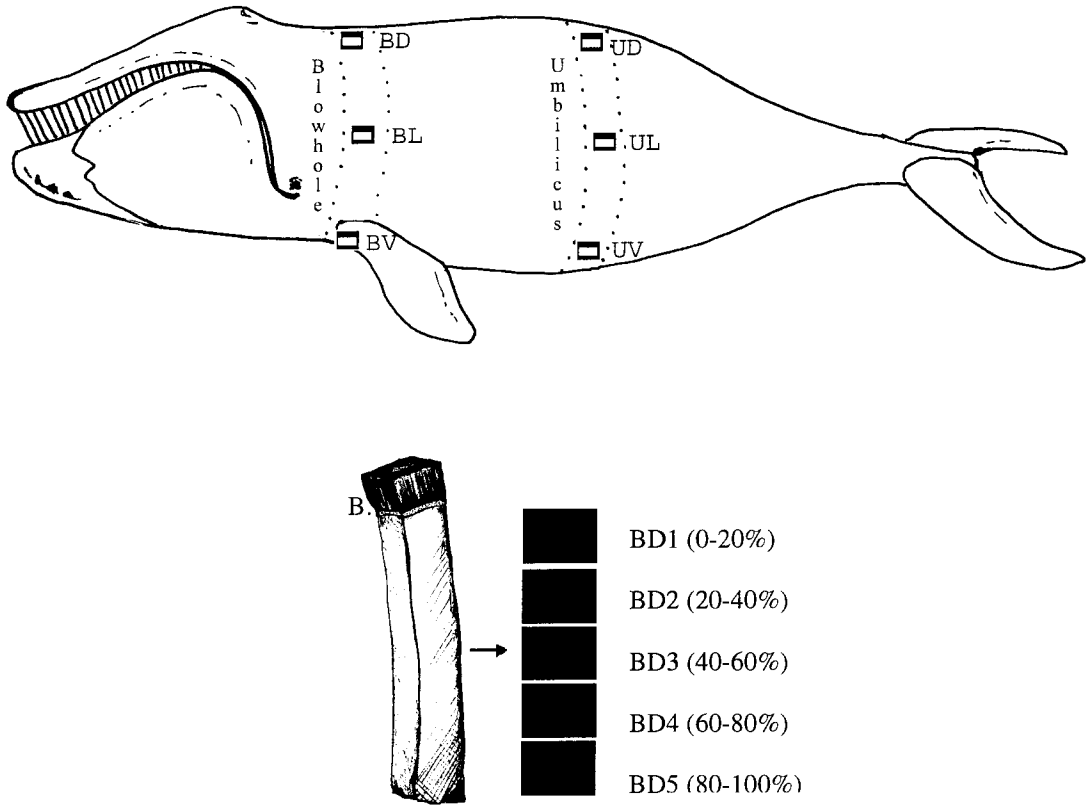
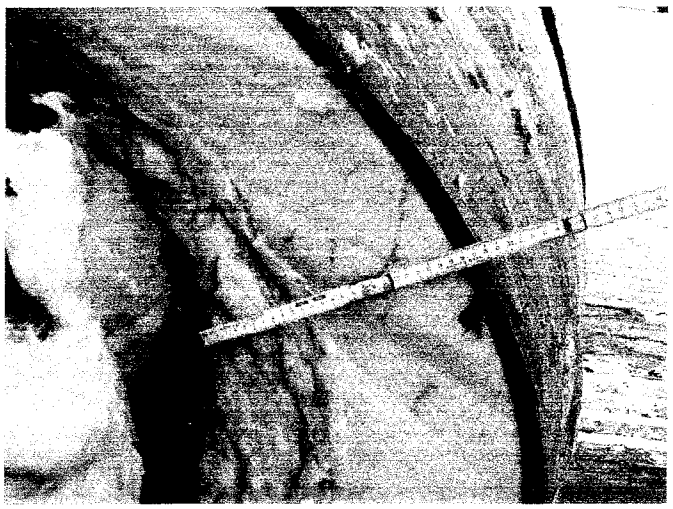
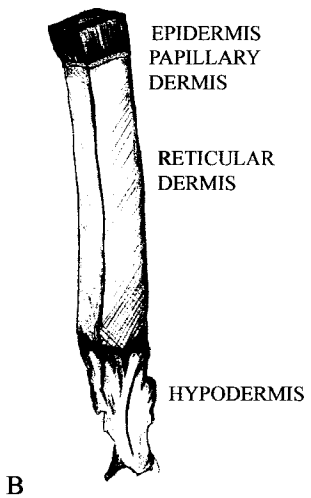


Figure 7.1. Blubber sampling scheme on the bowhead whale. (A) Areas of core sampling: anatomic sites are designated: BD (blowhole dorsal), BL (blowhole lateral), BV (blowhole ventral), UD (umbilicus dorsal), UL (umbilicus lateral) and UV (umbilicus ventral). Blubber at all six of these sites was sampled at depths 1-5 for blubber collagen percentage. Blubber at the three blowhole girth sites (BD, BL and BV) was sampled at depths 1,3 and 5 for percent total lipid. (B) Each core had the epidermis removed and the remaining reticular dermis divided into five equal parts for analysis (each 20% of the core).



A



B

Figure 7.2. Blubber layers in the bowhead whale (gross). (A) Photo illustrating the presence of a distinct hypodermal layer internal to the more structured reticular dermis. Arrow indicates the separation between reticular dermis and hypodermis. Note the inconsistency in the thickness of this layer due to the position and weight distribution of the whale. (b) Illustration depicting the different layers of blubber and hypodermis found in the bowhead whale (*Balaena mysticetus*).

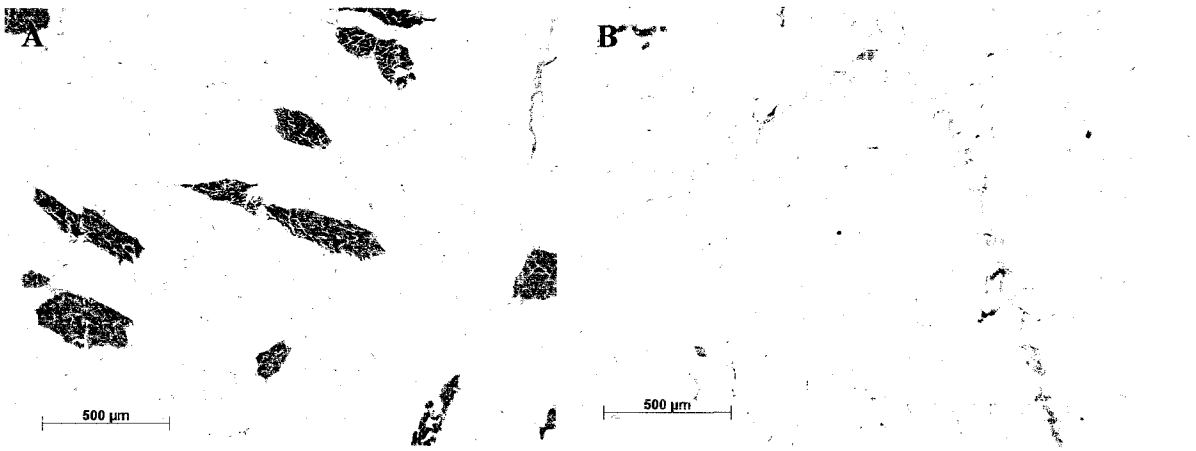


Figure 7.3. Photomicrographs of blubber from the bowhead whale. (A) Sample taken from the reticular dermis, depth 1 (BD1) and (B) a sample of hypodermal tissue take from an adult bowhead whale (*Balaena mysticetus*). Note the lack of collagenous structure in the hypodermal tissue.

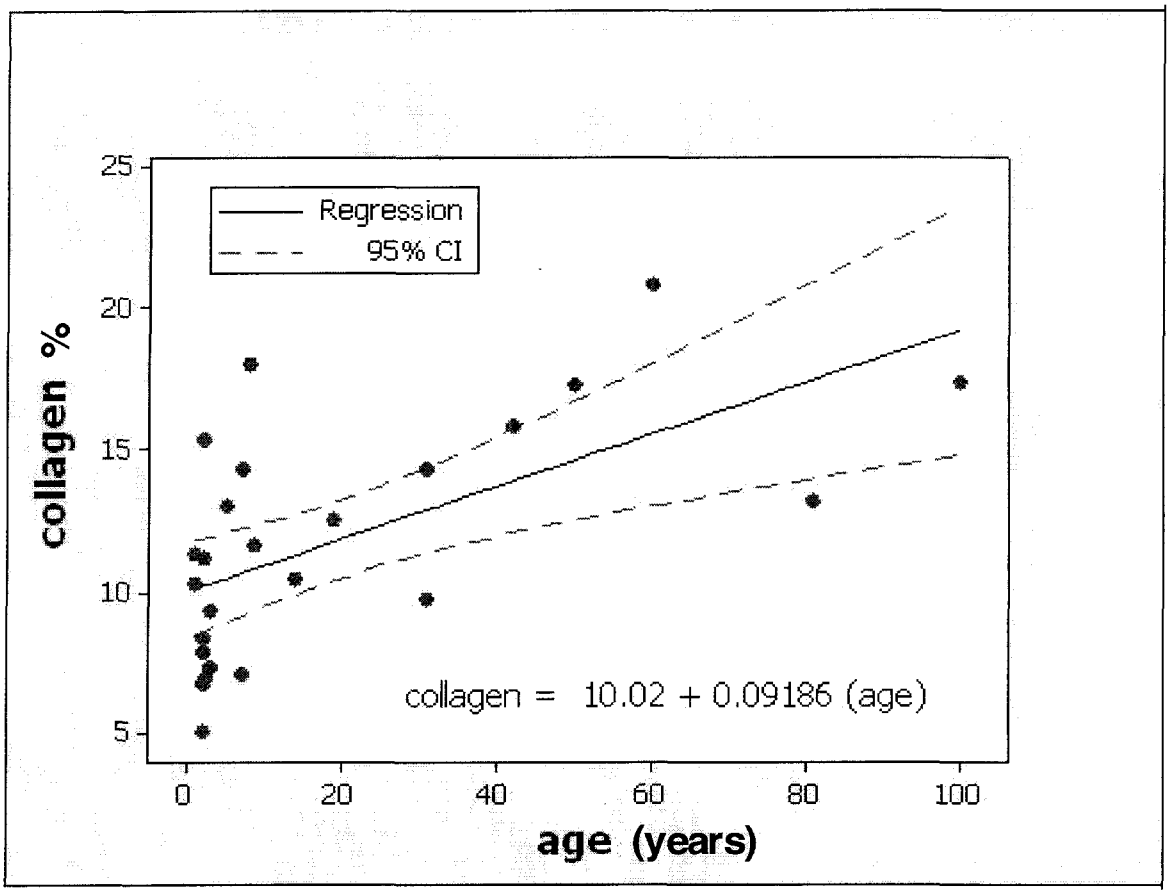


Figure 7.4. Simple linear regression illustrating the positive relationship between age (in years) and average blubber collagen percentage (BCP) in the bowhead whale.

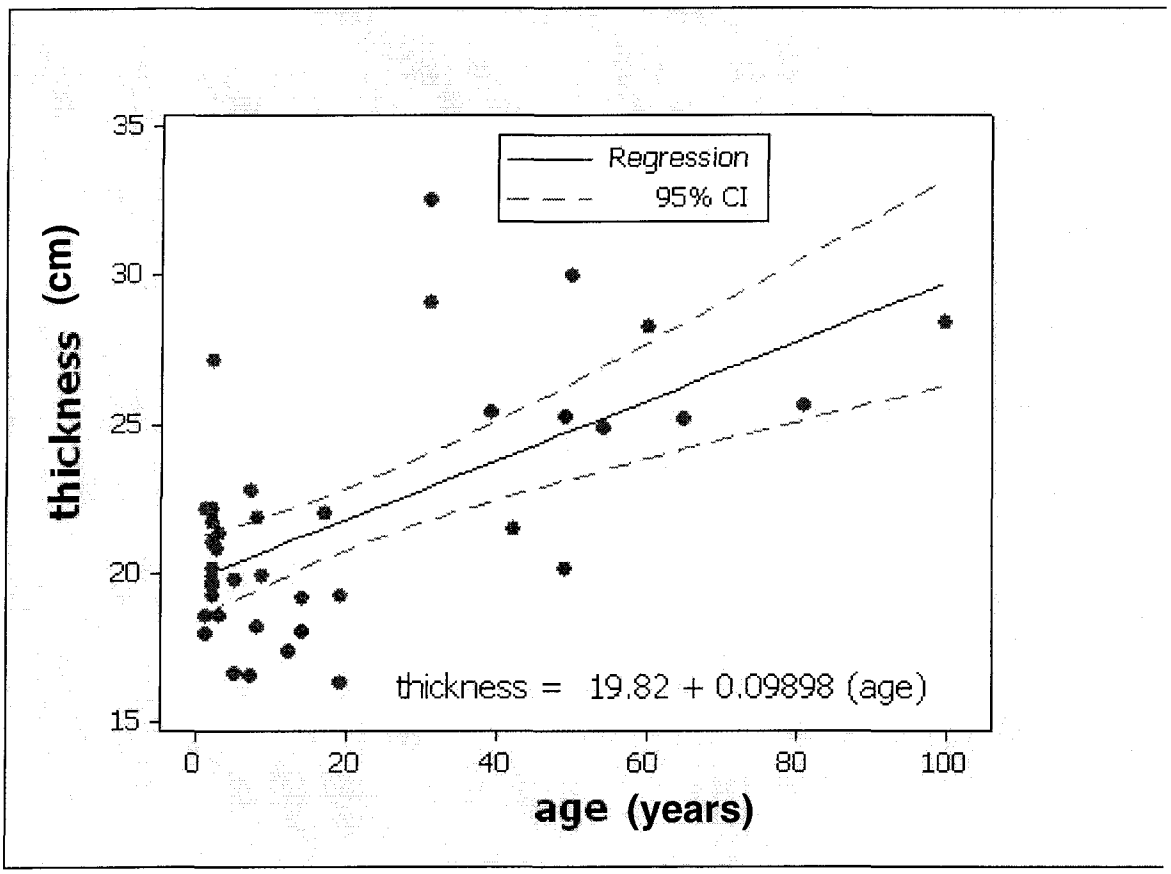


Figure 7.5. Simple linear regression illustrating the relationship between age (in years) and blubber thickness (cm) in the bowhead whale.

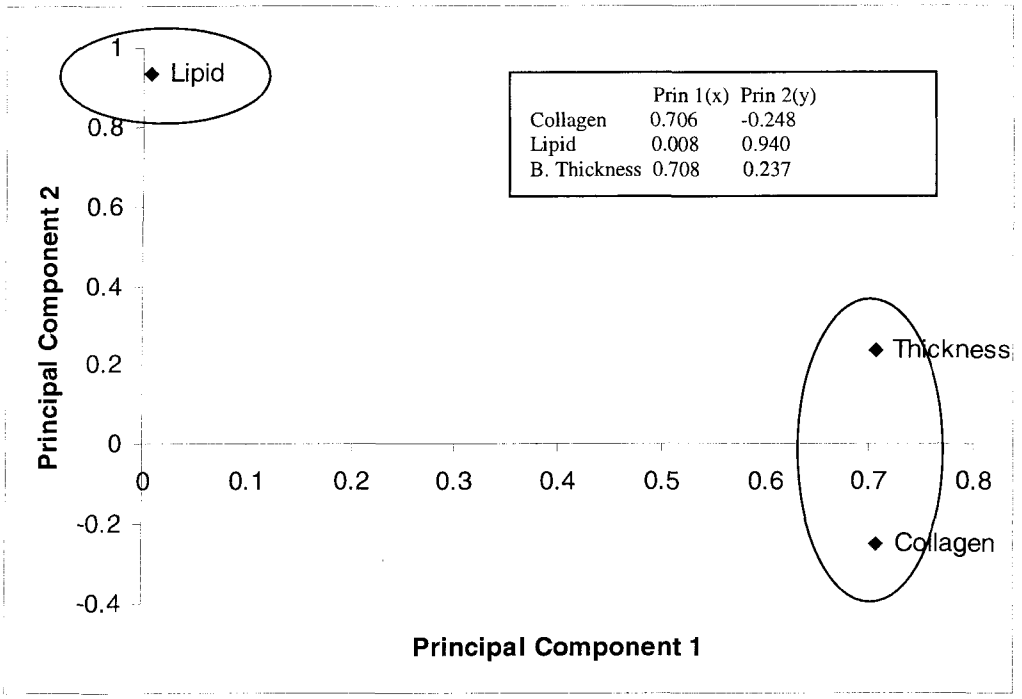
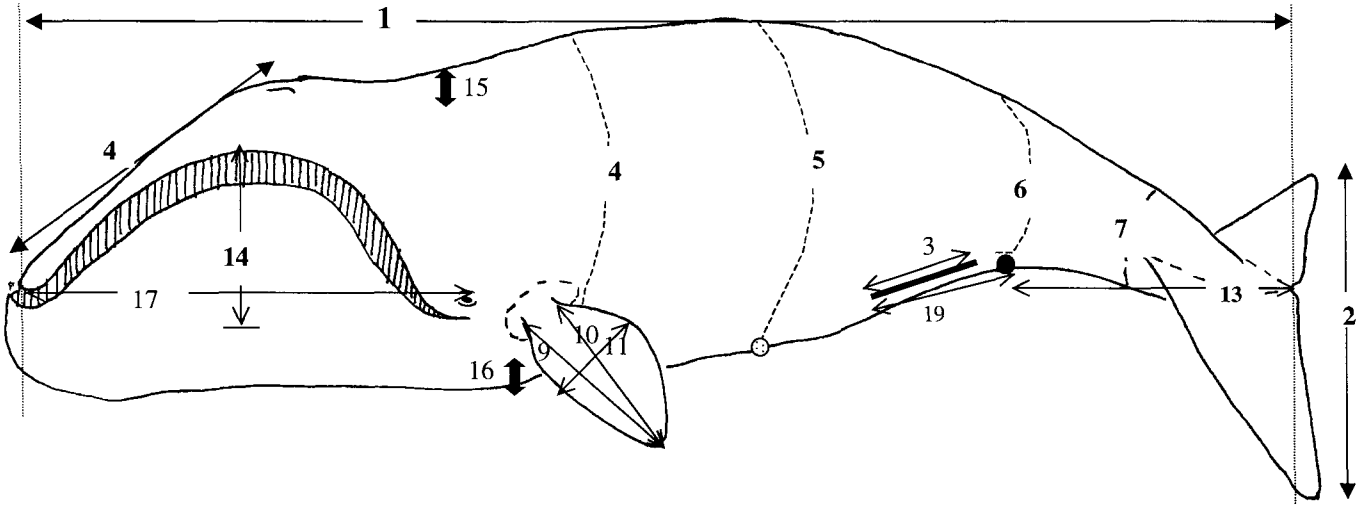


Figure 7.6. Principle components analysis evaluating the relationship between blubber collagen percentage, percent total lipid and blubber thickness (cm) in the bowhead whale.

Whale ID: _____ **Date:** _____



All measurements in **centimeters except total length!**

1. **Total length (feet only)** _____
2. **Fluke width** _____
3. **Genital slit length** _____
4. **Girth at axilla** _____
5. **Girth at umbilicus** _____
6. **Girth at anus** _____
7. **Girth at peduncle** _____
8. **Snout to blowhole** _____
9. **Ant. flipper length** _____
10. Post. flipper length _____
11. Flipper width _____
12. Hypodermis thickness ventral (on whale) _____
13. Fluke notch to anus _____
14. **Length of longest baleen** (to gum) _____
15. **Blubber thickness dorsal**[1] _____
(15) **Hypodermis thickness dorsal**[2] _____
16. **Blubber thickness ventral**[1] _____
17. Snout to eye _____
18. Snout to umbilicus _____
19. Ant. end of genital slit to anus _____

[1] Taken off the whale laying on the ground
[2] Taken ON the whale (thickness of hypodermis)

7.7. Appendix. Harvest diagram of morphological measurements taken from landed whales during the subsistence hunt (NSB-DWM).

Chapter Eight

Update on age estimation of bowhead whales (*Balaena mysticetus*) using aspartic acid racemization[7]

8.0 Abstract

Ninety-eight eye globes (from 84 individual bowhead whales) were collected and analyzed to estimate ages of the whales using the aspartic acid racemization aging technique. Racemization rate (k_{Asp}) was based on data from earlier studies of humans and fin whales; the estimate used was 1.175×10^{-3} yr $^{-1}$. The D/L ratio at birth $(D/L)_0$ was estimated using eyes from two near full-term bowhead fetuses. The $(D/L)_0$ value was 0.02708. Its variance, as well as the variance of the D/L ratios measured for whales older than age 0, was calculated via analysis of variance using multiple measurements from the same whale. Age estimates for each whale and standard errors (SE) of these age estimates were obtained using the delta method. Estimated ages of five individuals, all males, exceeded 100 years of age. The SE increased with estimated age, but the age estimates had lower coefficients of variation for older animals. The SE of the age estimates could be reduced by improving the laboratory protocol for determining the D/L ratio. Gross and histologic changes indicative of aging have been found in the >200 bowheads examined but do not appear to adversely affect health, and there is a striking lack of evidence for senescence for the many gonads examined. This, along with the recovery of "traditional" whale hunting tools from five recently harvested whales, suggests that life spans in excess of 100 years may be possible.

Keywords: age determination, bowhead whale, age at sexual maturity

[7] Rosa, C., Blake, J.E., George, J.C., Zeh, J., Botta, O., Zauscher M., Bada, J.L., O'Hara, T.M. Update on age estimation of bowhead whales (*Balaena mysticetus*) using aspartic acid racemization. Prepared for submission to the Journal of Cetacean Research and Management

8.1 Introduction

Accurate and precise age estimates are crucial to interpreting many aspects of marine mammal health, biology and population assessment. Valid estimates of the ages of sexual maturity, senescence and life span are critical to population modelers. In the field of health assessment, age can affect many organismal characteristics ranging from tissue chemical composition to the gross and histological appearance of tissues.

The age of a marine mammal may be determined by various methods, ranging from photo re-identification to such methods as ear plug growth layer measurement, tooth growth layer group quantification, aspartic acid racemization in the teeth or eye lens nucleus and, to a limited extent, the aging of baleen. For a review of these methods, see Christensen *et al.* (1981), Schell *et al.* (1989) and Hohn *et al.* (1989).

In mysticete whales, specifically the bowhead whale (*Balaena mysticetus*), estimating age is challenging. Teeth are not present, ear plugs do not appear to form and baleen aging is reliable only up to approximately eleven years of age due to wear at the distal ends of the baleen plates (Schell *et al.*, 1989). Recent research (aspartic acid racemization) has found that this species of whale may routinely live over 100 years of age, with data showing ages possibly in excess of 200 years (George *et al.*, 1999). Aspartic acid racemization (AAR) aging of the eye lens nucleus is the only published method, to date, for directly estimating ages of bowheads over 12m in length (Schell and Saupe, 1993; George *et al.*, 1999).

Briefly, the aspartic acid racemization aging technique is based on the fact that amino acids exist in two different optical isomers (the D and L enantiomers), which rotate plane polarized light in opposite directions, but at equal absolute values. Living organisms biochemically produce mainly the L-enantiomers of the amino acids, which is important for the functionality of enzymes, for example (Bada *et al.*, 1980). They maintain the disequilibrium state by continuous biosynthesis during metabolism. In the absence of metabolic activity, in tissues such as teeth and eye lens proteins in mammals, a chemical process called "racemization" begins immediately after the animal is born (or even earlier in its fetal stage). In the racemization reaction, the L amino acids are converted interchangeably into the D enantiomer and vice versa until both enantiomers have equal concentrations, i.e., a D/L ratio of 1.0 (this is called a racemic

mixture). The rate at which racemization occurs varies for each amino acid and is also temperature dependent, with higher temperatures leading to a higher reaction rate. Aspartic acid is an amino acid with a high racemization rate and can be used for age determination in the range of tens of years (humans, dolphins and other cetaceans). It should be noted that the D/L value of amino acids even in metabolically inactive fetal tissue ($(D/L)_0$) is not zero and must therefore be determined in order to use this technique. In most mammals, the core body temperature averages ~ 37°C. Since the racemization rates k_{AA} for amino acids such as aspartic acid (k_{Asp}) have been determined for this temperature from a sample of mammals with known ages and body temperature (Masters *et al.*, 1977), the age of other animals can be estimated from the D/L ratio, assuming they have a similar body temperature (Bada *et al.*, 1980). If the body temperature is not known accurately, educated estimates for the racemization rates can be used.

As part of a larger health assessment study, the ages for 42 whales (landed by Inuit hunters between 1998 and the year 2000) have been determined by aspartic acid racemization of their eye lens nuclei via methods developed by George *et al.* (1999). These data have been added to the previously analyzed bowhead data from George *et al.* (1999) (n=42) and the combined data (n=84) reanalyzed.

The objective of this study was to extend the previous work, to further evaluate the aspartic acid racemization aging method in cetaceans via analysis and comparison of paired eyes from individual whales and, finally, to provide essential data for the bowhead whale health assessment project.

8.2 Materials and Methods

8.2.1 Sample collection and preparation

Each eye (intact globe) was collected during the Inuit bowhead whale subsistence hunt in Alaska between 1978 and 2000. Eyes were frozen immediately after collection and shipped by airfreight to Scripps Institution of Oceanography (SIO). Eye lenses were dissected from the globes (via sterile technique) and the lens nuclei identified and separated from the surrounding lens tissue. Each lens nucleus from eyes collected between 1998 and 2000 was split in half, and the two halves were stored in an Eppendorf vial and a sterilized glass tube, respectively, at –20°C. The halves that were stored in the glass tubes were processed to determine D/L ratios of aspartic acid, while those in the Eppendorf vials are still archived.

8.2.2 Hydrolysis and estimation of D/L ratio of aspartic acid

1ml of doubly distilled (dd) 6 M HCl was added to each of the lens splits in the glass tubes to hydrolyze the protein, and the tube was flame sealed using a torch. The tubes were placed in an oven at 100°C for 6h. Under these conditions, no racemization of aspartic acid is expected. After removal, the vials were broken and placed in a centrifuge evaporator to remove the HCl under vacuum. Each of the residues was taken up in 1ml dd water and stored at –20°C. A variety of colors and turbidities was observed for the resulting solutions. The aspartic acid D/L ratios for eyes collected in 1978-1981 were determined from the solutions using ion-exchange chromatography (Nerini, 1983; Bada, 1984). All other aspartic acid D/L ratios were determined using high performance liquid chromatography (HPLC) (Zhao and Bada, 1995). Some further details regarding the processing of the sample solutions from the eyes collected between 1998 and 2000 are given in the following sections.

8.2.3 Desalting

Following unsuccessful experiments to analyze the raw extracts (interference with the derivatization reaction), the solutions were purified with an analogous method as used routinely for the desalting acid-hydrolyzed meteorite and sediment extracts. The sample solutions were added to a BIO-RAD AG® 50W-X8 cation exchange resin (prepared in sterilized Pasteur pipettes) and rinsed with dd water. The amino acids that remained on the resin were then eluted with 3ml 2 M NH_4OH solution into small glass tubes. These purified lens extracts were dehydrated under vacuum, and the residues were taken up in 200 µl dd water and stored at –20°C until analysis.

8.2.4 Derivatization and HPLC analysis

To 10 µl of the sample solutions were added 10 µl of 0.4 M Borate buffer, and this solution was dried under vacuum to remove traces of ammonia remaining from the purification procedure. Then 20 µl of dd water was added, followed by 5 µl of OPA/NAC reagent (Zhao and Bada, 1995). After one minute derivatization time, the reaction was quenched with 475 µl acetate buffer (pH 5.5); 50µl of the resulting solution were injected into the HPLC column. The signals for D- and L-aspartic acids were identified by

comparison with retention times of known standards. One standard (D/L = 0.08) was analyzed before and after each analysis session in order to assess the consistency of the system.

A Phenomenex *Luna* phenyl-hexyl-column (250 x 4.60mm) including a guard system was used in the analysis. The pump was a Hitachi L-6200 HPLC pump with low-pressure mixing. Eluents: Buffer A: Methanol; Buffer B: 50 mM sodium acetate buffer, pH 8. Elution was isocratic on buffer B at the retention time of aspartic acid, and buffer A was used to rinse the column after the analysis. The detector was a Shimadzu RF-530 fluorescence detector. The data were automatically integrated on a Hitachi D-7500 integrator. These raw data were used to calculate the D/L ratios of aspartic acid in the samples (see Appendix A).

8.2.5 Calibration

To calibrate the measured D/L ratios for aspartic acid, a set of standards was analyzed. First, a pure 10 mM solution of L-aspartic acid was prepared and analyzed to check for D-contamination in the purchased batch. No contamination was detected. Then, 1 mM solutions of aspartic acid were prepared with the following D/L ratios: D/L = 0.20, 0.15, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01. Each of these standards was injected three times, and the averages of the calculated D/L ratios were compared to the known values of the solutions. Based on these data, a calibration equation was determined by linear regression (R = 0.99617). The resulting equation is

$$(D/L)_{act} = 1.3219 \times (D/L)_{meas} + 0.0031$$

where $(D/L)_{act}$ is the actual D/L ratio and $(D/L)_{meas}$ is the measured one (Figure 8.1). This equation was then used to calculate the actual D/L ratios in the samples. These actual D/L values were used as in George *et al.* (1999) to determine the ages of the whales, as described in the next section.

8.2.6 Estimating age and the relationship of length to age

The actual D/L ratios described in the previous section were used in estimating age from the following equation:

$$\text{age} = [\ln((1 + D/L)/(1 - D/L)) - \ln((1 + (D/L)_0)/(1 - (D/L)_0))] / [2k_{Asp}]$$

where k_{Asp} is the racemization rate for aspartic acid, and $(D/L)_0$ is the D/L value at age 0. Ages for whales with both eyes sampled were estimated as the average of the age from the left eye and the age from the right eye. In one case in which we had two measurements from each eye, we were able to identify one as an outlier and omit it.

Growth rates were estimated by fitting the von Bertalanffy growth curve model to the data using nonlinear least squares. The fitted model was:

$$\text{length} = (L_{max} + I_F L_{diff})\ [1.0 - \exp(-(k + I_F\ k_{diff})\ (t - t_0))]$$

where t = age, L_{max} = average maximum length for males, I_F is coded as 0 for males and 1 for females, L_{diff} = average difference between female and male maximum length, k = the growth rate constant for males, k_{diff} = the difference between male and female growth rate, and t_0= age at length 0. Note that t_0 is not really an age but rather a constant analogous to the intercept in a linear model which permits the growth curve to be fit even if some of the age estimates are negative and the smallest lengths are much greater than zero.

8.2.7 Estimating the racemization rate for aspartic acid

The racemization rate (k_{Asp}) for aspartic acid is determined by regressing the natural log of (1+D/L)/(1-D/L) for a sample on the animals' ages (Masters *et al.*, 1977). The slope of the regression line estimates $2k_{Asp}$. In the case of humans and fin whales, the relationship appears to be linear. Masters *et al.* (1977) reported $2k_{Asp}$ for humans, with body temperature 37°C, as 2.50×10^{-3} yr^{-1} with a standard error (SE) of 0.29×10^{-3}yr^{-1} based on a sample of 17 normal human eye lenses. George *et al.* (1999) determined $2k_{Asp}$ for fin whales based on the sample of 16 whales given in Nerini (1983) (with average D/L used when two samples were available for the whale) as 2.209×10^{-3}yr^{-1} with SE = 0.716×10^{-3}yr^{-1}. Note that the maximum deep body temperature of fin whales (36.1° C) is lower than that of humans, and one would expect k_{Asp} to be lower (Brodie and Paasche, 1985). However, ages of the fin whales, based on ear plug data, are less

precisely determined than human ages, increasing the variability of the estimate of $2k_{Asp}$ for fin whales and perhaps leading it to be negatively biased. As in George et al. (1999), the human and fin whale estimates were averaged to allow for the possibility that whale $2k_{Asp}$ is lower than human. The resulting estimate of $2k_{Asp}$ is $2.35 \times 10^{-3} yr^{-1}$ with a variance $V_k = 0.149 \times 10^{-6} yr^{-2}$ and a standard error SE = $0.39 \times 10^{-3} yr^{-1}$.

8.2.8 Determining the $(D/L)_0$ (age 0) value

The $(D/L)_0$ value is a critical variable in calculating the age estimates. Because they had an eye lens from only one bowhead fetus, George *et al.* (1999) estimated $(D/L)_0$ and its variance V_0 using D/L measurements from all the sampled bowhead whales assumed on the basis of their body length and baleen length to be age 2 or less. An eye from an additional term fetus was collected in 1999 and used with the D/L value from the earlier fetus to estimate $(D/L)_0$. The D/L values for the two fetal eye lenses analyzed were 0.0270 and 0.0272. These two values, together with an additional D/L measurement from the second fetus, were used in an analysis of variance of $\ln((1 + D/L)/(1 - D/L))$ with between-whale and within-whale variance components to estimate V_0.

8.2.9 D/L ratio measurement error

To estimate the measurement error variance, paired D/L ratio measurements from 21 whales were analyzed. The data did not suggest that between-eye variability was higher than within-eye variability, so we did not distinguish in this analysis between measurements from different eyes and repeated measurements from the same eye. The residual mean squared error from an analysis of variance of $\ln((1 + D/L)/(1 - D/L))$ with between-whale and within-whale variance components provided an estimated variance V of a single measured value of $\ln((1 + D/L)/(1 - D/L))$.

8.2.10 Standard error for age estimates

As in George *et al.* (1999), we applied the delta method (Seber 1982, pp. 7-8) and a formula of Goodman (1960) for an estimate of the variance of a product of two independent random variables to calculate standard errors for the age estimates. This approach is necessary to treat the multiple sources of error in the estimates discussed above: measurement errors in the D/L ratios of the samples, variability in the D/L ratio at age 0, and error in the estimate of $2k_{Asp}$.

The equation for age given above can be written as the product of two independent random variables: age = xy where $x = [\ln((1 + D/L)/(1 - D/L)) - \ln((1 + (D/L)_0)/(1 - (D/L)_0))]$ and $y = 1/[2k_{Asp}]$. These quantities are clearly independent since x is estimated from bowhead data and y from human and fin whale data. Since the two terms in x are independent for all the sampled whales except the two term fetuses, for which we do not estimate age since it is defined as zero, an estimate of the variance of x is given by $V_x = V + V_0$. The delta method gives us $V_y = V_k/[2k_{Asp}]^4$ as an estimate of the variance of y. Then formula (5) of Goodman (1960) gives

$$V(\text{age}) = x^2V_y + y^2V_x - V_xV_y$$

where V(age) is the estimated variance of the age estimate with the appropriate measured and estimated quantities used in computing x and y. The SE is just the square root of V(age).

8.3 Results

8.3.1 Sampled bowheads

Eye lenses were collected from 84 bowhead whales. A distribution of sex and body length is given in Figure 8.2. Of these 84 whales, 46 (55%) were female and 38 (45%) were male; 31 (37%) were greater than 13m long and probably sexually mature. Fourteen of the 84 had paired right and left eye lenses analyzed.

We could not use a random sampling scheme to obtain the eyes used in this analysis. Eye globes were collected as whales were available. A hunt-based bias toward younger animals was expected. The International Whaling Commission (IWC) recommended that hunters should avoid taking mature animals in the early years of sampling. In addition, there is some hunter preference for smaller animals. However, the 37% estimated to be sexually mature in our sample does not differ greatly from the percentage of mature animals in the general population estimated from aerial photogrammetry at Barrow (41%, Angliss *et al.*, 1995).

8.3.2 Ages and growth

Estimated ages for all 84 whales are given in Table 8.1. Results of the von Bertalanffy model fit to the age-length data are given in Table 8.2 and Figure 8.3.

The estimated ages varied considerably for a given length class. This is expected for young animals (<11m) based on work by Schell *et al.* (1989). However, variability in k_{Asp} estimates, measured D/L ratios and $(D/L)_0$ values also produce substantial error (Table 8.1). This variability in the k_{Asp} rate is a major contributor to uncertainty, particularly in the older ages. D/L ratio measurement error accounts for most of the variability in the age estimates at the younger ages. In combination, these sources of error lead to large coefficients of variation (CV) for the age estimates, particularly for young animals. SE increased with age (Figure 8.4) while CV decreased; thus older animals have greater precision in a relative sense.

None of the whales <11m long had estimated ages that were implausible given their SE, with the possible exception of 00B16. However, published ages for whales in this size range are 11 years or less (Schell *et al.*, 1989). We therefore omitted whales with ages <1 (all of whom were <10m long) as well as all whales <10.5m long with ages of 20 or more from the von Bertalanffy fit. The fetuses were omitted because exploratory analyses indicated that the rapid growth in the first year of life could not be fit well. The estimated growth curve summarized in Table 8.2 indicates that females differ from males both in maximum length (17.6m vs 15.1m) and in growth rate. Females and males of the same age are estimated to be similar in length up to about age 10, but beyond this female lengths are estimated increasingly exceed male lengths. Sexual maturity – at 12.5 to 13m for males (O'Hara *et al.*, 2002) and 13 to 14m for most females (Koski *et al.*, 1993; George *et al.*, 2004) – is estimated to occur in the mid to late twenties.

8.3.3 Estimate of $(D/L)_0$

The mean value of $\ln((1 + (D/L)_0)/(1 - (D/L)_0))$ for the two term fetuses was 0.05418, corresponding to $(D/L)_0 = 0.02708$, close to the average of the fetal D/L values 0.0270 and 0.0272. The estimated variance of the $\ln((1+ (D/L)_0)/(1 - (D/L)_0))$ estimate was $V_0 = 0.4907 \times 10^{-4}$.

8.3.4 D/L ratio measurement error

There were 44 D/L ratio measurements from 3 fin (Nerini, 1983) and 18 bowhead whales (including left and right eye globes from 17 animals). A paired *t*-test between left and right globes for the bowhead whales indicated only a minor difference in D/L ratio between eyes (left eyes having a D/L ratio higher on average by 0.0055 than right eyes, $P = 0.02$). The residual mean square from an analysis of

variance of $\ln((1 + D/L)/(1 - D/L))$, with each whale with paired data constituting a group, was $V = 0.6075 \times 10^{-3}$. Thus the standard error of a single $\ln((1 + D/L)/(1 - D/L))$ measurement was 0.0246.

The measurement error variance for the whales sampled since 1996 was significantly higher than the corresponding estimate of George *et al.* (1999) (0.8385×10^{-3} versus 0.7946×10^{-4}, $P = 0.002$). This may be because the new pairs covered a broader range of D/L values. In addition, although most of the paired data values reflect relatively small measurement error, the new data pairs include three outliers with differences between the paired values 2 to 4 times as large as the differences in the other pairs. Such outliers could result from sample contamination. There were no such outliers in the paired data of George *et al.* (1999), perhaps because with fewer pairs, there was a lower probability of outliers being present. Pending further investigation of measurement errors, we opted to use the value of V given above for estimating standard errors of age estimates from both the old and new data.

8.4 Discussion

8.4.1 Growth, age at sexual maturity and longevity

George *et al.* (1999) reported growth, age at sexual maturity and longevity related topics with respect to the AAR technique based on 42 bowhead whales. Our results, with an addition of 42 whales, support these findings and will not be revisited in depth in this paper. In brief, the additional data support the findings of an initial high rate of growth following birth, followed by an interrupted period of growth after weaning. This period is succeeded by steady growth up until sexual maturity is attained, at 12.5-13m for males (O'Hara *et al.*, 2002) and 13-14m for females (Figure 8.3) (Koski *et al.*, 1993; Schell *et al.*, 1989; George *et al.*, 2004). This roughly correlates with 24-29 years of age in males and 23-30 years of age in females.

In addition, with the larger sample size, the results from the nonlinear least squares von Bertalanffy fit (Table 8.2) show that differences exist not only between the male and female L_{max} but also between the male and female k; all parameters had significant t values. This indicates that males and females differ in growth rate as well as in maximum length.

8.4.2 Viability of the AAR technique for aging whales

The AAR technique is most appropriate for whales over approximately 20 years of age. Age estimates below 26 in Table 8.1 had estimated SE = 11 unless both eyes were sampled so that an average with SE = 8 could be computed. As discussed below, improving the accuracy and precision of the HPLC analyses could reduce standard errors. Olsen and Sunde (2002) reported lower SE for AAR estimates of minke whale ages. For older bowhead whales, even the standard errors estimated from our data do not prevent the age estimates for individuals from being useful. Our AAR ages for young bowheads can be used in estimating life history parameters even though they provide little information about the ages of individuals. Progress is being made in the area of baleen aging for the younger whales (Lubetkin *et al.*, 2004).

8.4.3 Implications for cetacean health assessment studies

Age affects a majority of biological health parameters, including tissues (histological interpretation), toxicology (bioaccumulation of contaminants), reproduction and basic hematological parameters such as biochemistry panels and complete blood counts (which often differ between mature and immature members of mammalian species). The bowhead whale health assessment project depends upon AAR and baleen aging to accurately assess these parameters *with respect to age*. As an example, bowhead whales exhibit little pathology at a gross or microscopic level. After hundreds of necropsies and review of thousands of histology slides, few incidences of pathology have been noted. The discovery of renal (kidney) fibrosis in numerous whales, which can have a marked effect upon individual whale health, was a significant but yet unexplained finding. This change is thought to relate to age and increased renal cadmium concentrations (though Cd accumulates with age, as well) (Willetto *et al.*, 2002, Rosa, unpublished data). This is just one example of the importance of reasonably accurate age estimates to the interpretation of results. Another noteworthy example is the fact that, presently, there is no strong evidence of reproductive senescence in the bowhead whale. The implications of this are far-reaching and may be another unique feature of the bowhead whale among mammals.

8.4.4 Future improvements to AAR methodology / future research directions

From this analysis, we have several suggestions to improve cetacean AAR analysis. First, we recommend

the development of a standard laboratory protocol and certified standards of cetacean lens material. Samples should be analyzed in random order with no prior knowledge of body length, sex or reproductive status. Also, a clear protocol for repeat analyses of samples should be decided upon prior to data collection. Data from repeated runs for the 42 whales sampled most recently are available and will be used to design such a protocol. These data can also be used in estimating V for each eye analyzed instead of a single value applied to all eyes, permitting more accurate estimation of the SE of the age estimated from each eye. With an adequate number of repeated runs for each sample, statistical techniques for outlier detection can be used to eliminate discrepant measurements, and measurement error can be reduced considerably by averaging.

Second, we now have enough data to estimate $(D/L)_0$ only from term fetuses. However, efforts should be made to collect additional fetal eyes in order to strengthen the estimate.

Finally, samples that are of interest due to their utility in the process of aging cetaceans should be collected under a set protocol. This includes eyes, baleen (for aging young whales), collagen (for aging method development) and ovaries (for corpora counts).

8.4.5 Other techniques

In conjunction with this study, other methods of aging are being pursued in bowheads. Two promising approaches include baleen aging using stable isotopes (Lubetkin *et al.*, 2004) and collagen aging (Rosa *et al.*, 2001). These techniques may fill the gap that exists and provide ages for younger whales, where the standard errors tend to be too large to infer age from the AAR estimates. Additionally, as an adjunct method, standard counts of ovarian corpora albicantia and corpora lutea from harvested females can provide important reproductive information that can be useful in age determination. This technique is currently being investigated to assess its utility in bowhead whales (George *et al.*, 2004).

8.4.6 Problems with AAR age estimates

AAR age estimates (based on the nucleus of the lens) will over-estimate age if the animal has cataracts (brunnescent group IV) (Masters *et al.*, 1977). Cataracts have not been reported in bowhead whales (Philo *et al.*, 1993), and there was no evidence of cataracts noted during gross dissection of the eye lenses obtained in this study.

Another factor that could result in AAR-based ages being over-estimates would be the presence of more asparagine residues (in comparison to humans and other mammals, including several marine species) in the eye lens nucleus proteins of bowhead whales. Asparagine racemizes several times faster than aspartic acid (Geiger and Clarke, 1987; Brinton and Bada, 1995). Thus, if there are higher abundances of asparagine residues in the bowhead lens proteins, which are hydrolyzed to aspartic acid during 6 M HCl treatment, this would generate an apparently higher D/L ratio for aspartic acid. The apparent extent of aspartic acid racemization in the bowhead whale eye lens nucleus samples would thus be greater than in eye lens nucleus samples from other mammals of similar age. Using the human and fin whale based racemization rates would then give age estimates for bowhead whales that were too old. However, it seems unlikely that bowhead whales would differ from other cetacean species in this regard.

We recognize that specific aspartic acid residues in αA and αB-crystallin (lens proteins) racemize at different rates in humans which could lead to inaccuracies in age estimates (Fujii *et al.*, 1994a; Fujii *et al.*, 1994b). However, since the approach of Zhao and Bada (1995) measures the overall D/L value, the racemization rates should not differ significantly between bowhead whales.

The AAR age estimates would be biased *downwards* if: a) the average temperature experienced by the eyeglobe was lower than in humans or fin whales, or b) the samples were contaminated with blood or "modern" tissue. The cornea is in contact with very cold water throughout the year, and it is possible that the internal temperature of the globe is lower than deep body temperature. Sub-normal temperatures would slow racemization and subsequently the AAR aging technique would *under*-estimate age to some degree. If surrounding tissue or blood contaminates the sample (lens nucleus) during dissection, the D/L ratio could be dramatically lowered, resulting in a gross underestimate (George *et al.*, 1999).

Implications for bowhead whale management

As noted in George *et al.* (1999), the longevity of bowhead whales has relatively minor direct implications for the management of the aboriginal hunt by Alaskan Eskimos. Population abundance and trend and subsistence need are the principle factors in determining the quota. As background, the subsistence harvest of bowhead whales is regulated at international, national and local levels. The strike quota is established by the IWC (IWC, 1982) based upon the nutritional and cultural needs of the Eskimo

communities. In past years, quota level was estimated using assessment models under the provisions of Paragraph 13a of the International Whaling Commission (IWC) schedule (IWC, 1982). Currently, the quota request is evaluated by the *Bowhead SLA* (Strike Limit Algorithm) (IWC, 2003). The *Bowhead SLA* was developed by members of the IWC Scientific Committee (SC) and tested in trials, each simulating 100yrs of bowhead management, covering a broad range of assumptions about the bowhead population and subsistence hunts. The SC agreed that the *Bowhead SLA* is the "best tool for providing management advice for this stock" (IWC, 2003; p. 28). The *Bowhead SLA* determines whether the quota request can be met based on current and past population abundance and hunts (IWC, 2003). However, age information is taken into account in periodic *Implementation Reviews* that evaluate whether the status of the population and hunt is within the range tested in the trials.

8.5 Acknowledgments

We express our appreciation to the North Slope Borough Department of Wildlife Management, the Cooperative Institute for Arctic Research (CIFAR), the University of Alaska Fairbanks (UAF)/Experimental Program to Stimulate Competitive Research (EPSCoR) and NOAA for generous funding and logistical support. We would also like to thank the Inupiat subsistence hunters in Barrow and Kaktovik for graciously allowing us to collect these samples. Individual thanks to Victoria Woshner, Gay Sheffield, Lara Dehn, Cyd Hans, Benjamin Akootchook, Chris Terzi, and Stephen Brown for their help with the manuscript, tissue collection and analytical support. Bowhead whale samples were collected under the authority of permit #932-1489-00 for the MMHSRP program (Dr. Teri Rowles). Support for this project came from CIFAR/NOAA (NSB BU3613) and Alaska INBRE grant number 2 P20 RR016466 from the National Center for Research Resources, National Institutes of Health, Dept. of Health

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Table 8.1.

Basic data for sampled bowhead whales with age estimates determined using aspartic acid racemization. Whale identification number indicates: year, village (B = Barrow, G = Gambell, WW = Wainwright, H = Pt. Hope, S = Savoonga) and sequential harvest number. Age and its standard error (SE) were set to zero for the two fetuses used in D/L_0 calculations. All other age estimates were estimated from D/L ratios given in Appendix A, with SE obtained by the delta method. When D/L ratios from both eyes were available, the two age estimates were averaged, resulting in a smaller SE. Females (F) are shown on the left and males (M) on the right. NA = not available.

Whale id	Sex	Length (m)	Baleen (cm)	Age	SE	Whale id	Sex	Length (m)	Baleen (cm)	Age	SE
99B18F	F	4	11	0	0						
95B8	F	4.1	10	0	0						
96B2	F	7.6	67	9	11						
99KK1	F	7.7	85	1	11						
00B9	F	7.9	70	6	8						
99B19	F	8.1	96	1	8	96B15	M	8.1	70	4	11
78B1	F	8.5	146	3	11	78B3	M	8.4	95	-5	11
96B1	F	8.5	126	20	11	94B14	M	8.4	76	0	11
95B4	F	8.6	102	2	11	79B1	M	8.7	75	0	11
00B8	F	8.6	NA	2	11	94B11	M	8.7	151	2	11
00KK3	F	8.8	112	1	11	99B24	M	8.8	NA	9	11
00B15	F	8.9	NA	7	8	00B1	M	8.9	NA	3	11
00B18	F	8.9	NA	10	11	99B5	M	9	NA	-1	11
99B20	F	9	130	5	8	79H3	M	9.1	105	-2	8
99B12	F	9.2	174	7	11	93B18	M	9.3	200	9	11
00KK1	F	9.2	150	7	11	99B9	M	9.3	193	6	11
78H1	F	9.3	150	-2	11	00B13	M	9.4	189	-4	11
00B10	F	9.4	166	9	11	00B17	M	9.5	NA	9	8
98B25	F	9.7	NA	35	12	78H2	M	9.7	164	8	11
99B22	F	9.7	175	9	8	99B21	M	10.5	174	10	8
00B14	F	9.9	191	6	11	00B12	M	10.8	193	13	8
00B16	F	10	NA	45	13	99B23	M	10.9	192	12	11
96B18	F	10.1	202	23	11	96B16	M	11	212	27	12
96B24	F	10.9	243	23	8	99B8	M	11	217	8	11
98B12	F	11.3	174	8	11	96B22	M	11.6	199	23	8
96B9	F	12.1	240	32	12	00KK2	M	12.1	214	34	12
95B12	F	12.3	NA	11	11	96B19	M	12.9	249	32	12
99B6	F	12.6	236	22	11	95B11	M	13	263	20	11
96B6	F	12.7	235	31	12	96B17	M	13.3	269	35	12
99B18	F	13	342	29	12	78G1	M	13.8	298	23	8
96B10	F	13.4	320	26	12	00B11	M	13.8	NA	60	14
96B4	F	14.4	300	43	13	95B16	M	14.1	NA	92	19
00B2	F	14.5	273	26	8	99B13	M	14.1	303	46	13
79WW1	F	14.6	316	58	14	99B14	M	14.2	NA	57	14
00B3	F	14.6	NA	41	13	95B15	M	14.5	289	58	14
99B16	F	14.8	NA	39	12	95WW5	M	14.6	NA	213	36
95B10	F	15	320	20	11	99B15	M	14.6	NA	70	16
99B7	F	15.4	NA	71	16	80S1	M	14.7	229	48	13
00B4	F	15.4	NA	52	14	00B6	M	14.7	232	17	11
81G1	F	15.5	297	35	12	99B17	M	14.9	NA	113	22
80G1	F	15.6	291	27	12	78WW2	M	15	319	136	25
81WW1	F	16.2	NA	29	12	95B7	M	15.2	305	160	28
78WW1	F	16.3	322	70	16	95B9	M	17.4	384	174	30
95B13	F	16.5	NA	48	13						
81S1	F	16.8	NA	39	12						
00B5	F	18.9	331	66	15						

Table 8.2. Results of von Bertalanffy curve fit for the bowhead whale.

L_{max} = average maximum length for males, k = growth rate constant for males, t_0 = age at length 0;
L_{diff} = average difference between female and male maximum length, so
$L_{max} + L_{diff}$ = average female maximum length;
k_{diff} = difference between male and female growth constant, so
$k + k_{diff}$ = growth rate constant for females.

Parameter	Value	SE	*t*-value
t_0	-16.23	3.34	-4.86
L_{max} (male)	15.07	0.42	36
k (male)	0.0439	0.0072	6.10
L_{diff}	2.551	1.025	2.49
k_{diff}	-0.0099	0.0047	-2.11

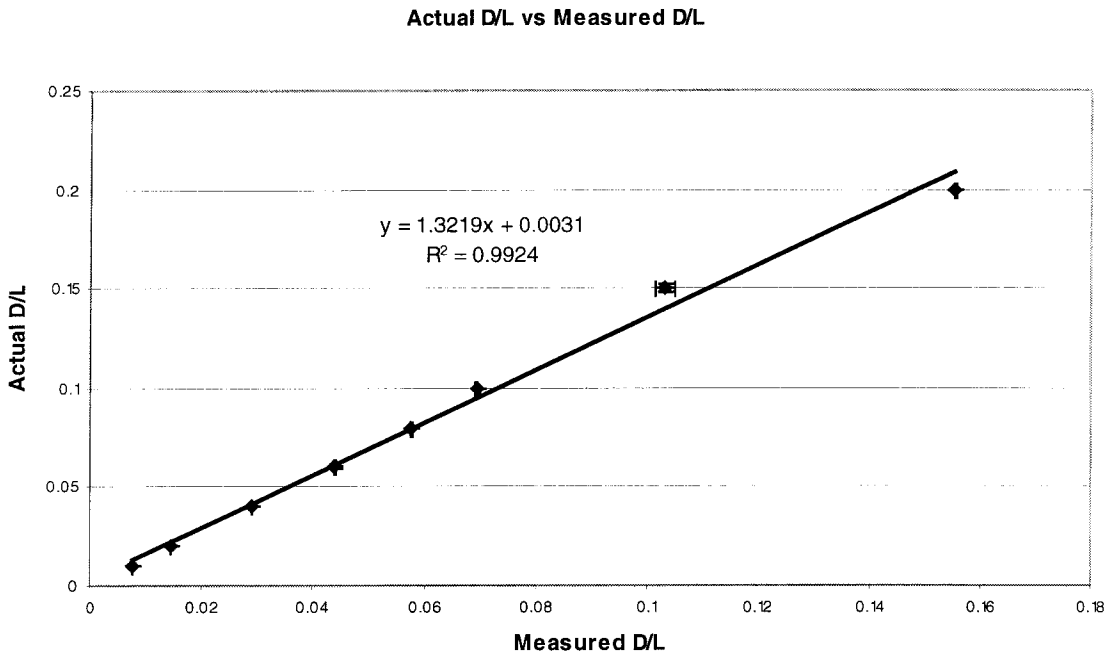

Figure 8.1. Linear regression of the measured D/L ratios versus the actual values for the standard aspartic acid solutions used to calibrate the sample data for the bowhead whale samples analyzed (n=84).

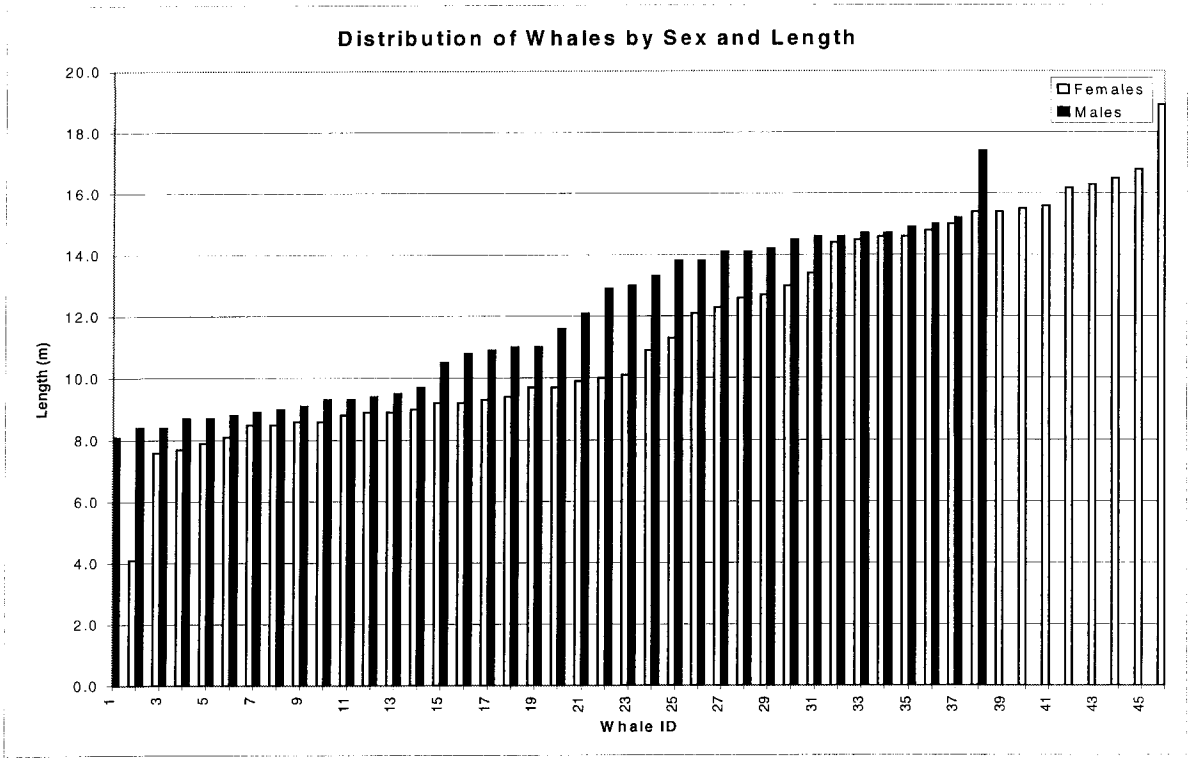

Figure 8.2. Distribution of sex and body length of bowhead whales sampled for AAR analysis.

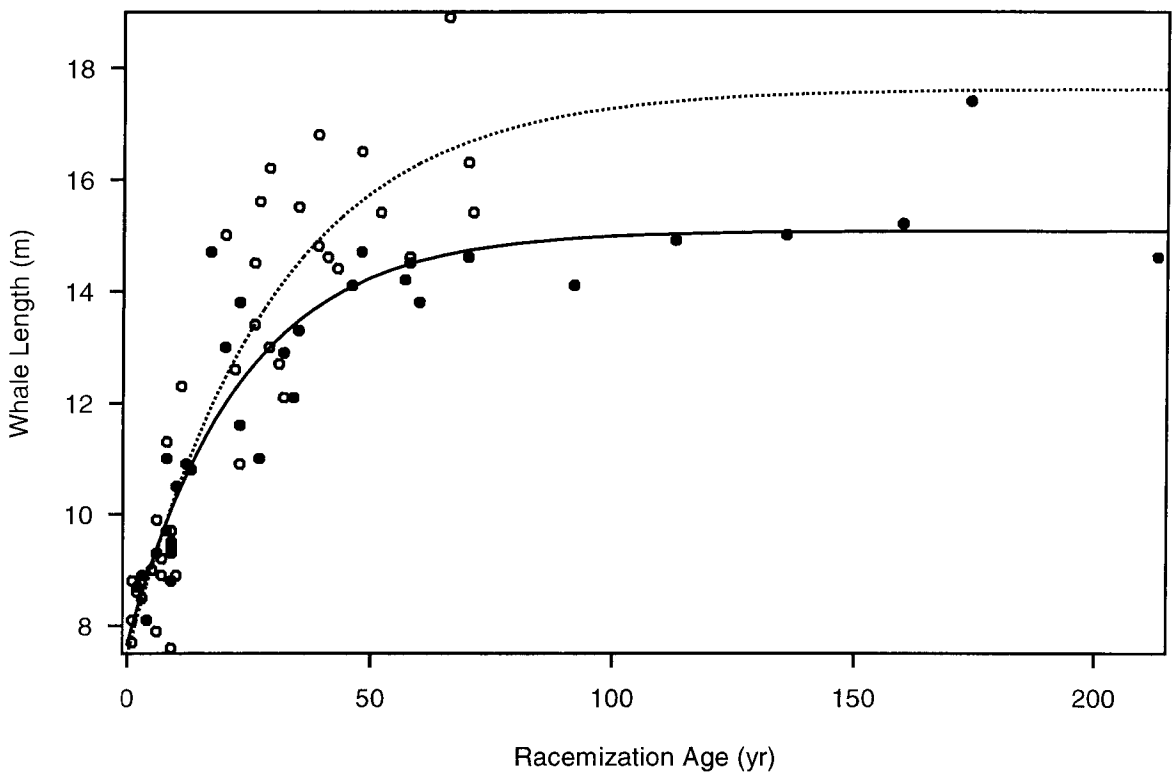

Figure 8.3. Estimated age-at-length for bowhead whales using the aspartic acid racemization technique. The von Bertalanffy growth curves are shown for females (upper curve) and males (lower curve).

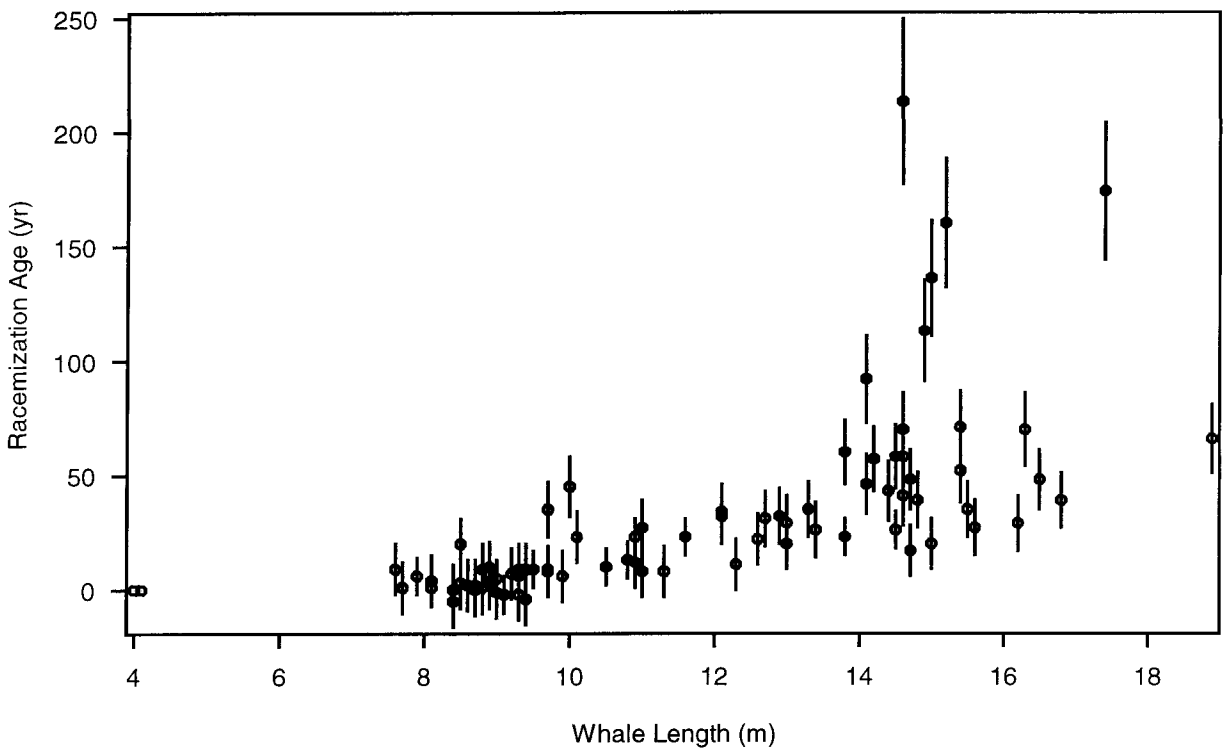

Figure 8.4. Age estimates (yr) by bowhead whale length (m) shown with estimated standard errors. Note that the standard error increases with age. Open circles females, closed circles males.

8.7 Appendix

Basic data for bowhead whale eyes used in this study with age estimates determined using aspartic acid racemization. Whale identification number indicates year, village and sequential harvest number; F indicates a fetus. Info is L (left), R (right), **A** (unknown whether left or right), **B** (second eye in pair with orientation unknown), 1 (first measurement on the eye if there are two measurements), 2 (second measurement on the eye). The D/L ratios for the samples obtained in the 1978-1981 harvest were determined using ion exchange chromatography (Bada, 1984); all other D/L ratios were determined using the HPLC based method (Zhao and Bada, 1995). NA = not available.

Whale id	Info	Length (m)	Sex	Baleen (cm)	D/L	Village	Age (yr)	SE
78B1	A	8.5	F	146	0.031	Barrow	3.2	10.8
78B3	L	8.4	M	95	0.022	Barrow	-4.7	10.8
78G1	L	13.8	M	298	0.051	Gambell	20.4	11.3
78G1	R	13.8	M	298	0.057	Gambell	25.4	11.5
78H1	A	9.3	F	150	0.025	Point Hope	-2.1	10.8
78H2	R	9.7	M	164	0.037	Point Hope	8.4	10.8
78WW1	L	16.3	F	322	0.109	Wainwright	70.1	15.8
78WW2	R	15.0	M	319	0.185	Wainwright	136	24.8
79B1	L	8.7	M	75	0.027	Barrow	-0.1	10.8
79H3	L	9.1	M	105	0.026	Point Hope	-0.9	10.8
79H3	R	9.1	M	105	0.024	Point Hope	-2.6	10.8
79WW1	A	14.6	F	316	0.096	Wainwright	58.5	14.4
80G1	A	15.6	F	291	0.059	Gambell	27.2	11.6
80S1	R	14.7	M	229	0.083	Savoonga	47.7	13.3
81G1	A	15.5	F	297	0.068	Gambell	34.9	12.2
81S1	A	16.8	F	NA	0.072	Savoonga	38.7	12.5
81WW1	R	16.2	F	NA	0.062	Wainwright	29.4	11.8
93B18	R	9.3	M	200	0.038	Barrow	9.3	10.9
94B11	R	8.7	M	151	0.030	Barrow	2.5	10.8
94B14	R	8.4	M	76	0.027	Barrow	-0.1	10.8
95B10	L	15.0	F	320	0.051	Barrow	20.4	11.3
95B11	A	13.0	M	263	0.051	Barrow	20.4	11.3
95B12	A	12.3	F	NA	0.040	Barrow	11.0	10.9
95B13	A	16.5	F	NA	0.083	Barrow	47.7	13.3
95B15	L	14.5	M	289	0.095	Barrow	58.0	14.4
95B16	L	14.1	M	NA	0.135	Barrow	92.5	18.6
95B4	A	8.6	F	102	0.030	Barrow	2.5	10.8
95B7	R	15.2	M	305	0.212	Barrow	160	28.4
95B8F	A	4.1	F	10	0.027	Barrow	-0.1	10.8
95B9	R	17.4	M	384	0.227	Barrow	174	30.5
95WW5	A	14.6	M	NA	0.270	Wainwright	213	36.5
96B1	A	8.5	F	126	0.051	Barrow	20.4	11.3
96B10	R	13.4	F	320	0.058	Barrow	26.4	11.6
96B15	R	8.1	M	70	0.032	Barrow	4.2	10.8
96B16	R	11	M	212	0.059	Barrow	27.2	11.6
96B17	R	13.3	M	269	0.068	Barrow	34.9	12.2
96B18	R	10.1	F	202	0.054	Barrow	22.9	11.4
96B19	L	12.9	M	249	0.065	Barrow	31.9	12.0
96B2	A	7.6	F	67	0.038	Barrow	9.3	10.9
96B22	L	11.6	M	199	0.058	Barrow	26.4	11.6
96B22	R	11.6	M	199	0.049	Barrow	18.7	11.2
96B24	L	10.9	F	243	0.057	Barrow	25.5	11.5
96B24	R	10.9	F	243	0.052	Barrow	21.2	11.3
96B4	R	14.4	F	300	0.077	Barrow	42.6	12.8

Whale id	Info	Length (m)	Sex	Baleen (cm)	D/L	Village	Age (yr)	SE
96B6	L	12.7	F	235	0.063	Barrow	30.6	11.9
96B9	R	12.1	F	240	0.065	Barrow	32.3	12.0
98B12	A	11.3	F	174	0.037	Barrow	8.2	10.8
98B25	A	9.7	F	NA	0.068	Barrow	34.6	12.2
99B5	L	9.0	M	NA	0.026	Barrow	-1.1	10.8
99B6	L	12.6	F	236	0.053	Barrow	21.9	11.3
99B7	L1	15.4	F	NA	0.110	Barrow	70.9	15.9
99B7	L2	15.4	F	NA	0.059	Barrow	27.5	11.7
99B8	R	11.0	M	217	0.037	Barrow	8.3	10.8
99B9	R	9.3	M	193	0.035	Barrow	6.5	10.8
99B12	L	9.2	F	174	0.036	Barrow	7.4	10.8
99B13	R	14.1	M	303	0.081	Barrow	46.3	13.2
99B14	L	14.2	M	NA	0.094	Barrow	57.3	14.3
99B15	A	14.6	M	NA	0.109	Barrow	70.0	15.7
99B16	L	14.8	F	NA	0.072	Barrow	38.6	12.5
99B17	A1	14.9	M	NA	0.159	Barrow	113	21.5
99B17	A2	14.9	M	NA	0.173	Barrow	125	23.2
99B18F	A1	4.0	F	11	0.027	Barrow	0.1	10.8
99B18F	A2	4.0	F	11	0.022	Barrow	-4.1	10.8
99B18	A1	13.0	F	342	0.061	Barrow	28.9	11.8
99B18	A2	13.0	F	342	0.050	Barrow	19.7	11.2
99B19	L	8.1	F	96	0.031	Barrow	3.7	10.8
99B19	R	8.1	F	96	0.025	Barrow	-1.9	10.8
99B20	L	9.0	F	130	0.045	Barrow	15.4	11.0
99B20	R	9.0	F	130	0.020	Barrow	-6.1	10.8
99B21	L	10.5	M	174	0.039	Barrow	10.6	10.9
99B21	R	10.5	M	174	0.037	Barrow	8.8	10.9
99B22	L	9.7	F	175	0.043	Barrow	13.3	11.0
99B22	R	9.7	F	175	0.032	Barrow	4.4	10.8
99B23	R	10.9	M	192	0.042	Barrow	12.3	10.9
99B24	A	8.8	M	NA	0.037	Barrow	8.6	10.8
99KK1	L	7.7	F	85	0.028	Kaktovik	0.9	10.8
00B1	L	8.9	M	NA	0.031	Barrow	3.2	10.8
00B10	L1	9.4	F	166	0.079	Barrow	44.7	13.0
00B10	R1	9.4	F	166	0.037	Barrow	8.6	10.8
00B10	L2	9.4	F	166	0.039	Barrow	9.9	10.9
00B10	R2	9.4	F	166	0.033	Barrow	4.9	10.8
00B11	A	13.8	M	NA	0.097	Barrow	59.5	14.5
00B12	L	10.8	M	193	0.044	Barrow	14.3	11.0
00B12	R	10.8	M	193	0.040	Barrow	10.8	10.9
00B13	A	9.4	M	189	0.022	Barrow	-4.1	10.8
00B14	R	9.9	F	191	0.035	Barrow	6.4	10.8
00B15	A	8.9	F	NA	0.031	Barrow	4.1	10.8
00B15	B	8.9	F	NA	0.038	Barrow	9.3	10.9
00B16	R	10	F	NA	0.079	Barrow	44.6	13.0
00B17	L	9.5	M	NA	0.037	Barrow	8.4	10.8
00B17	R	9.5	M	NA	0.038	Barrow	9.2	10.9
00B18	R	8.9	F	NA	0.039	Barrow	10.1	10.9
00B2	L	14.5	F	273	0.060	Barrow	28.3	11.7
00B2	R	14.5	F	273	0.054	Barrow	22.9	11.4
00B3	R	14.6	F	NA	0.075	Barrow	40.7	12.7
00B4	A	15.4	F	NA	0.088	Barrow	51.7	13.7
00B5	R	18.9	F	331	0.103	Barrow	65.5	15.2
00B6	L	14.7	M	232	0.047	Barrow	16.6	11.1
00B8	R	8.6	F	NA	0.030	Barrow	2.5	10.8

Whale id	Info	Length (m)	Sex	Baleen (cm)	D/L	Village	Age (yr)	SE
00B9	L	7.9	F	70	0.035	Barrow	6.5	10.8
00B9	R	7.9	F	70	0.032	Barrow	4.5	10.8
00KK1	L	9.2	F	150	0.037	Kaktovik	7.3	10.8
00KK2	R	12.1	M	214	0.067	Kaktovik	34.2	12.1
00KK3	L	8.8	F	112	0.028	Kaktovik	0.7	10.8

Chapter Nine

Collagen aging in the bowhead whale (*Balaena mysticetus*)[8]

9.0 Abstract

Age in marine mammals may be determined by various means, ranging from photo re-identification to such methods as ear plug and tooth growth layer measurement, aspartic acid racemization in the teeth and eye lens nuclei, and the isotopic aging of baleen. Bowhead whales (*Balaena mysticetus*) are among the longest-lived mammals on earth, living to estimated ages well in excess of 100 years. In this species, teeth are not present, ear plugs do not appear to form and baleen aging is reliable only up to eleven years of age due to wear at the distal ends of the baleen plates.

This study evaluates the potential of aging whales via the analysis of a small amount of skin (from a biopsy dart sample or collection at necropsy). Manifestations of aging are most pronounced in the extracellular matrix (ECM), which is made up primarily of collagen. Skin undergoes dramatic age-related changes in its mechanical properties, including changes in tissue hydration and resiliency. Collagen cross-linking increases with age, and advanced glycation end-products, such as pentosidine and carboxy-methyl lysine (CML), accumulate in long-lived tissue proteins. Methods employed as indicators of aging include measuring the level of pentosidine and other collagen-related chemicals in the skin. Pentosidine, a marker of glycoxidative stress in skin collagen, increases as collagen ages in several terrestrial species that have been studied. It is one of the advanced glycation end-products (AGEs) of the Maillard reaction and an indicator of the extent of chemical modification, oxidation and cross-linking of tissue protein caused by reducing sugars. Pentosidine has been measured in mammalian skin via extraction, hydrolyzation and HPLC.

We quantified pentosidine and other collagen related products from the dermal collagen of 47 bowhead whales. Pentosidine is present at very low levels in bowhead whale dermal collagen suggesting that bowhead whale collagen does not age appreciably, that advanced glycation end-products in marine mammals differ from those found in terrestrial mammals or that alternative biochemical methods must be

[8] Rosa, C., Blake, J.E. Collagen aging in the bowhead whale (*Balaena mysticetus*). Prepared as "note" for submission to the Journal of Cetacean Research and Management

developed to quantify pentosidine in this species. Further research is needed to clafify the value of pentosidine quantification as an aging method in the bowhead whale.

9.1 Introduction

In bowhead whales (*Balaena mysticetus*), teeth are not present to be evaluated, ear plugs do not appear to form and baleen aging is reliable only up to approximately eleven years of age due to wear at the distal end of the baleen plates. Recently, aspartic acid racemization of the lens nucleus in bowhead whales has been successfully employed as an aging technique (George et al., 1999, Rosa et al., 2005). These analyses indicate that bowhead whales may routinely live to over 100 years of age and that their maximum life span may exceed 150 years of age. However, the collection of eye lenses is possible only in deceased whales. Additionally, this method is not optimal in whales less than twenty years of age due to the large error values generated in associated statistical analyses (baleen isotopic carbon analyses are a more appropriate aging method in these younger whales) (Lubetkin et al., 2004). Terrestrial mammals produce advanced glycation end-products (AGEs) at a rate proportionate to aging (Sell et al., 2000). Researchers have measured AGEs in the skin of several species of known-age mammals and have evaluated AGE quantification as a potentially valuable means of aging these species. The ability to age whales via the analysis of a small amount of skin may exist.

The extracellular matrix (ECM) exhibits the most aging changes, and is composed largely of collagen (Kohn, 1982). Skin undergoes dramatic age-related changes in its mechanical properties, including changes in tissue hydration and resiliency (Carino et al., 2000). Collagen cross-links steadily increase with age (Bailey et al., 1998). It is this fact that forms the basis of much of the human research in this field. One of the methods employed as an indication of aging is measuring the level of pentosidine (an AGE) in the skin and blood.

Pentosidine, a marker of glycoxidative stress, forms in skin collagen in a curvilinear fashion, increasing with age. This rate is inversely related to maximum life span across several mammalian species including the dog, cow, rhesus and squirrel monkey, least shrew, miniswine, Fisher 344 rat and man (Sell et al., 2000). Pentosidine is one of the advanced products of the Maillard reaction (characterized by the production of heavily cross-linked, advanced glycosylation end-products derived from Amadori products) and is a cross-link consisting of arginine, lysine and a pentose sugar. The formation of pentosidine involves both glycation and oxidation and serves as an indicator of the extent of chemical modification, oxidation

and cross-linking of tissue protein caused by reducing sugars (Bailey, 2000, Yamauchi, et al., 1988). Dermal pentosidine can be measured via a process of extraction, hydrolyzation and high performance liquid chromatography (HPLC). This work has the potential to provide a useful method of aging in living cetaceans, contributing essential life history data in this species and possibly other cetacean species, some of which are endangered.

Our objective was to determine the viability of pentosidine quantification as a method of aging in marine mammals. Measurement of pentosidine was undertaken in the bowhead whale with hopes of creating an aging curve with data drawn from whales ages via apartic acid racemization (AAR) technique.

9.2 Materials and Methods

9.2.1 Sample Collection

Samples of papillary dermis were collected from bowhead whales (n=47) during the Inuit subsistence hunt over a three year period (1998-2000). At the time of necropsy, skin samples (epidermis, dermis and hypodermis) were taken and frozen initially at -20°C at the Arctic Research Facility in Barrow, Alaska (USA) and subsequently at -80 °C at the University of Alaska Fairbanks, Fairbanks (USA). Samples used were taken from a dorsal area on the whale, approximately three meters caudal to the blowhole. These sample collections were conducted with permission of the Barrow Whaling Captain's Association and the Alaska Eskimo Whaling Commission through the Department of Wildlife Management (North Slope Borough, Alaska) under the purview of a National Oceanographic and Atmospheric Association (NOAA) permit (permit #932-1489-00 and 932-1489-03 for the MMHSRP program).

9.2.2 Sample Processing

Samples (2.5 g) from the papillary dermis (the area of tough collagen just below the epidermis) were minced and then homogenized for 15 minutes with a Brinkman Polytron-Aggregate homogenizer in 10 ml of phosphate buffered saline (PBS), pH 7.4. The insoluble collagen was recovered by centrifugation (DuPont Sorvall, Newtown, Connecticut USA) at 14000 RPM, 20,229 x g for 20 minutes at 4°C. The collagen was then extracted for 24 hours at 4°C in chloroform/methanol 2:1, followed by extraction in 1 M sodium chloride (pH 7.4) for 24 hours, 0.5 M acetic acid for 48 hours and finally pepsin at 0.1 mg/ml/0.5

M acetic acid for 24 hours. Mixtures were agitated during all extraction procedures. The insoluble collagen was centrifuged (DuPont Sorvall) at 1200 RPM for 20 minutes and washed three times in water and freeze-dried.

9.2.3 Preparation of tissue hydrolysate

Five mg of freeze-dried dermal collagen was placed into a 13x100 mm screw-capped tube. A total of 2 ml deaerated 6M HCl was added to the tube. The tube was then purged and "topped" with nitrogen and sealed with a Teflon-faced rubber lined cap. All samples were acid hydrolyzed for 24 hours at 110°C. The acid was evaporated by a Speed-Vac (Savant Instruments, Holbrook, New York USA) and each sample was reconstituted with 1 ml of water containing 0.01 M heptafluorbutyric acid. After each sample was filtered through a 4-μm filter, collagen content was determined for all samples by hydroxyproline calorimetric assay assuming collagen content of 14% hydroxyproline by weight as described by Sell and Monnier (1989). In all cases, samples were equalized for hydroxyproline (to 250 μg/ml) by diluting the sample with water containing 0.01 heptafluorobutyric acid.

9.2.4 HPLC Analysis

Pentosidine was determined by a repetitive injection technique as described by Odetti *et al* (1992). Samples of 100μl volume equivalent to 45 μg of hydroxyproline were injected into a C_{18} reversed-phase analytical column (Vydex 0.46 x 25 cm, 10 μm; The Separations Group, Hesperia, California USA). The column was attached to a high-performance liquid chromatograph equipped with model 510 pumps, a model 712 WISP automatic injector, and a model 680 controller (Waters Corporation, Milford, Massachusetts USA). The pentosidine peak was monitored at excitation wavelength 335/emission wavelength 385 nm by an online JASCO 821-FP spectroplourometer (Jasco, Easton, MD USA). The column eluted with a gradient of 0-0.06 M NaCl in 0.02 sodium acetate over 40 minutes at a flow rate of 1.0 ml/minute. The fluorescence detector was interfaced to a computer loaded with Azur chromatography software for recording and integration of chromotagraphic peaks (Datalys, Saint Martin d'Heres, France). Pentosidine eluted at approximately 29 minutes and a pentosidine standard was run between every five bowhead whale samples.

9.2.5 Statistical analyses

All statistical analyses were performed on the SAS operating system (SAS Institute, Inc., SAS Campus Drive, Cary, NC USA). Values were considered significantly different at $P<0.05$. Samples (pentosidine and unknown substance eluting at ~14.8 mins.) were analyzed for gender, season, length and age significance, using necropsy findings and estimated age data generated via AAR (George et al., 1999, Rosa et al.,2005) and baleen carbon isotope analyses (Lubetkin et al., 2005). Ages were generated via these methods for 40/47 whales in this study. The seven whales for which we did not determine ages were not included in aging comparisons; however, the entire data set (n=47) was included in the length comparisons. Regression analyses were performed between length, estimated ages (AAR) and pentosidine and unknown substance concentrations. Unknown substance was defined as the substance eluting at or within 0.5 seconds of 14.85 minutes.

9.3 Results

Results showed little or no pentosidine to be present in the 48 samples tested (Table 9.1). The elution time for pentosidine was ~28 minutes. However, an unknown peak was noted to elute repetitively at 14.85 ±0.5 minutes in all but one of the samples (46/47) (Figure 9.1). The fraction that eluted from each sample during both the pentosidine standard and the unknown substance elution time frame was collected for later analysis. Both the pentosidine peak and the area under the integrated peak that formed at or near 14.85 minutes were regressed against length and aspartic acid racemization and baleen isotopic aging results. There were no significant increases with age or length noted (Figures 9.2 and 9.3) (Pentosidine, age: $R=0.15$, length: $R=0.06$, unknown substance, age: $R=0.01$, length: $R=0.06$). No correlation was found between pentosidine and the unknown substance. None of the values for pentosidine or the unknown substance varied significantly with gender or season of capture.

9.4 Discussion

The results of this study show negligible amounts of pentosidine in the dermal collagen of the 47 bowhead whales examined as compared to several other terrestrial mammalian species previously investigated (Sell et al., 2000). Pentosidine extracted from dermal collagen increased as a function of age in six of terrestrial mammalian species (Sell et al., 2000). However, we did not find this relationship in

bowhead whales. The lack of increase in pentosidine levels as a function of age in the bowhead whale samples may be related to a host of different factors.

Bowhead pentosidine accumulation rates may be controlled by inherited factors related to species specific skin biology, such as collagen turnover and/or the ability to withstand the potentially harmful effects of glycoxidation due to the Maillard reaction (Sell et al., 2000, Verzijl, 2000). Related to this idea, it is possible that the turnover of collagen in the dermis of this species may be so great throughout the organism's lifetime, or so unpredictable, that pentosidine does not accumulate as it does in terrestrial mammals. Bowhead whales have among the thickest "skin" found in the mammals. It can reach depths of >50 cm including epidermis, dermis and hypodermis (Burns 1993). Modifications to accommodate skin of this thickness could be necessary in order for normal function to occur. These modifications may involve an increased turnover of collagen that does not decrease in rate as the animal ages. Alternatively, the average temperature of the papillary dermis may differ from terrestrial mammals, resulting in altered results.

Oxidative stress occurs *in vivo* during aging and is considered to be a major cause of molecular damage to cellular and tissue structures (Odetti et al., 2001). Little is known about the antioxidant status of these whales. It is known that their skin is rich in vitamins A and E and that their diet is composed of prey that are high in poly-unsaturated fatty acids (PUFAs) (Burns, 1993, Ackman et al., 1970, Rosa, unpublished data). The effects of diet on aging in cetaceans warrants further investigation, as PUFAs and other dietary compounds may effect the amount of glycation that occurs in the ECM and/or interfere with the Maillard reaction providing the organism with a protective "anti-aging" effect (Garibaldi et al., 2001). Alternatively, these long-lived mammals may be producing very few AGEs in general as they age. This is the most intriguing of the possibilities, as these whales may live to be older than 150 years of age (George et al., 1999).

Glucose is an important mediator of the aging process (Cerami et al., 1985) and is known to join with proteins during the Maillard reaction, resulting in AGE production. To date, all serum samples analyzed via standard serum biochemical analyses have found these whales to be normoglycemic, as compared to other terrestrial and marine species (Rosa, unpublished data). However, bowhead whales are

believed to fast from late fall to early spring (Burns, 1993), and all blood samples in this study were gathered predominately during periods that whales were near or in potential feeding areas, which could result in increased or variable serum glucose levels due to the collection period. Glycated hemoglobin concentrations indicate the average glucose level over the previous ~100 days of life in mammals. The determination of glycated hemoglobin concentrations in this species may provide additional insight into the rate of AGE production by providing a more reliable index of average glycemia than blood tests taken at capture and analyzed via standard biochemical methods. This may make it possible to incorporate fasting blood glucose levels drawn from the 3 months prior to collection and may provide critical insight into the aging process in this long-lived species.

Finally, bowhead whales may have evolved a method that is unique or different from terrestrial mammals to deal with collagen cross-linking and AGE production. The resulting end-product produced may be a substance other than pentosidine, or the biochemical method of quantification used for terrestrial mammals may not be appropriate or effective in cetaceans, especially those with appreciable amounts of lipid in their skin. Additional biochemical analyses are needed to see if changing the analytical process will yield more fruitful results.

Future work should include pentosidine quantification studies on cetacean tissues that have a slower rate of turnover (such as articular cartilage) (Verzijl et al., 2001) and analysis of the eluted fractions collected in this study. The unknown substance that was detected at ~14.85 minutes on HPLC analysis also needs further investigation, though it did not increase with age, and no relationships (age, season, gender) were apparent.

9.5 Acknowledgements

We express our appreciation to the Department of Wildlife Management, North Slope Borough (Barrow, Alaska), the Cooperative Institute for Arctic Research (CIFAR), UAF INBRE (Idea Networks for Biomedical Research Excellence) and NOAA for generous funding and logistical support. We thank the Inupiaq subsistence whalers in Barrow and Kaktovik and the Alaska Eskimo Whaling Commission (AEWC) for graciously allowing us to access these samples. Individual thanks to Gay Sheffield, Victoria Woshner, Lara Dehn, Todd O'Hara, Mike Castellini, Doug DeMaster, Cyd Hans, Craig George, Benny Akootchook and Chris Terzi, for their help with the manuscript, tissue collection and analytical support. Bowhead whale samples were collected under the authority of permit #932-1489-00 for the MMHSRP program (Dr. Teri Rowles).

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Table 9.1. Mean pentosidine and unknown substance concentration present in the bowhead whales examined (n=47) as compared to pentosidine values in the dog, cow and pig (Sell et al., 2000). N/a = not available.

Age subgroup	mean pentosidine (pmol/mg of collagen)	mean unknown substance (pmol/mg of collagen)
Bowhead Whale (*Balaena mysticetus*)		
Adult	0.23 (12, 0.16)	7.36 (12, 2.63)
Subadult/Juvenile	0.20 (25, 0.16)	8.81 (25, 4.41)
Fetal	1.14 (3, 0.16)	13.92 (3, 14.0)
Dog (*Canis familiaris*)		
Subadult/Juvenile	~ 5	n/a
Adult	~ 30-40	n/a
Cow (*Bos taurus*)		
Subadult/Juvenile	~5	n/a
Adult	~20	n/a
Pig (*Suis scrofa*)		
Subadult/Juvenile	~ 2	n/a
Adult	~15	n/a

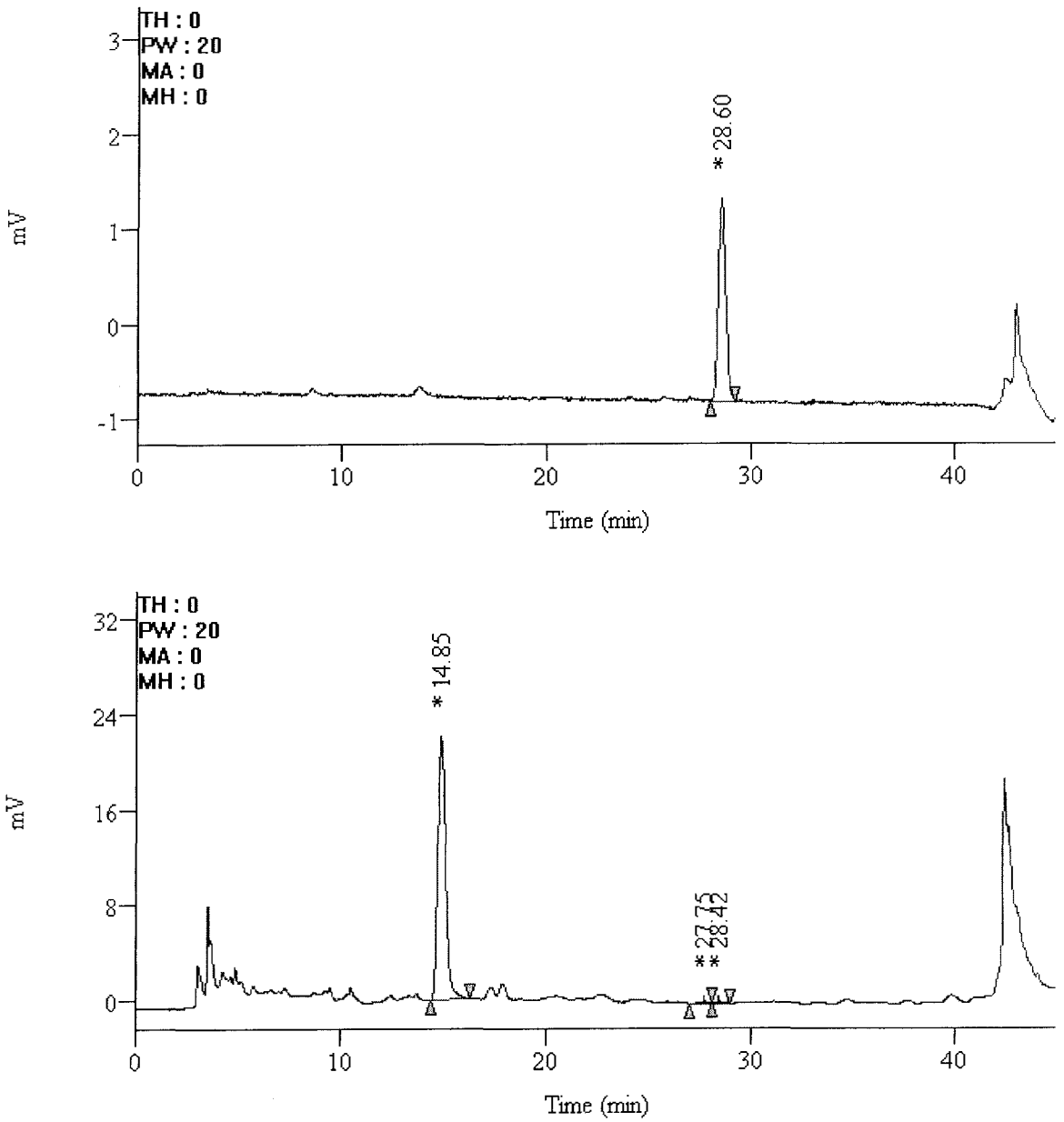


Figure 9.1. Chromatograms for (A) the pentosidine standard and (B) a typical bowhead whale dermal collagen sample. Note the elution spike at 14.85 minutes in the bowhead sample. This corresponds to the unknown substance fraction that was collected during HPLC analysis and was present in 46/47 whales analyzed.

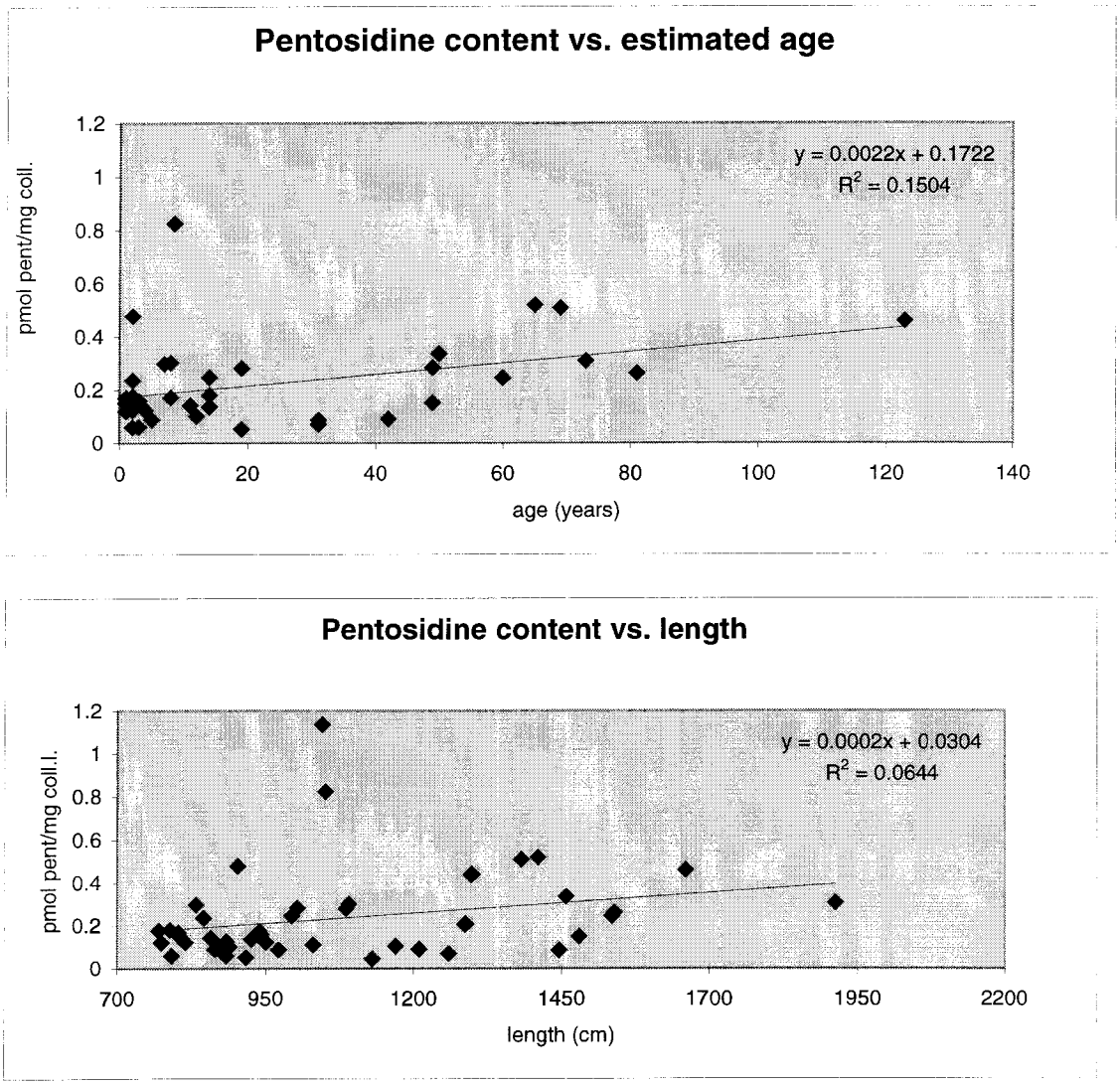
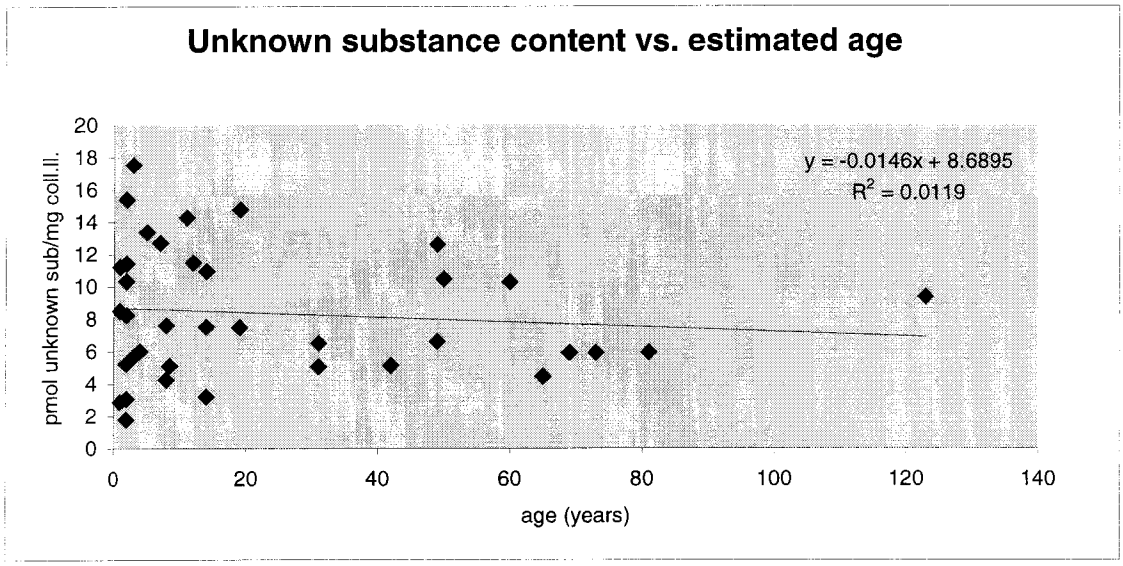


Figure 9.2. Pentosidine as a function of estimated age and length in skin biopsies (dermal collagen) from bowhead whales. (A) Age: y= 0.002x + 0.17, R=0.15, n=40. (B) Length: y= 0.0002x + 0.03. R=0.06, n=47.



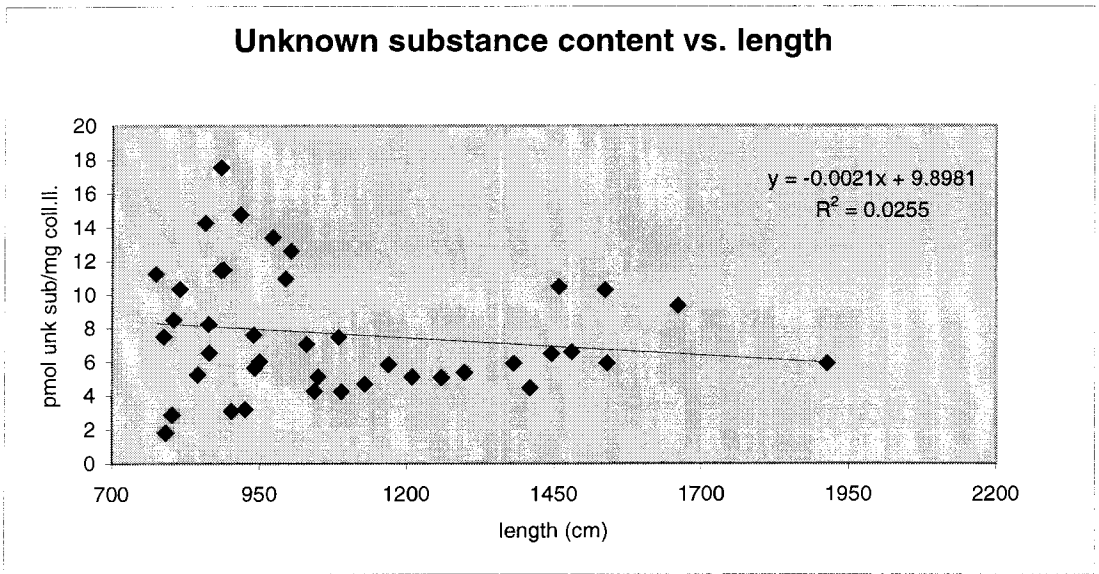


Figure 9.3. Unknown substance (eluting at ~14.85 minutes) as a function of estimated age and length in skin biopsies (dermal collagen) from bowhead whales. (A) Age: y=0.1461x + 8.69. R=0.01, n=40. (B) Length: y= 0.0021x + 9.90. R=0.03, n=47.

Chapter 10

General Conclusions

The whales included in this study were healthy compared to the many terrestrial and marine mammals I have examined in the past. Evidence of this includes a lack of lesions at the gross and light microscopic level, good to excellent body condition in almost all of the whales examined and the presence of positive population growth with a record number of cow-calf pairs in 2001 (George *et al.*, 2004). Unexpected observations in the bowhead whale include a high prevalence of splenic extramedullary hematopoiesis, hepatic lipidosis, renal interstitial fibrosis and pulmonary fibromuscular hyperplasia. Although there is considerable variability in the occurrence and severity of these unusual microanatomic features, none of these conditions appear to cause health problems. Given the reliance on comparative medicine when evaluating microscopic anatomy, the recognition of species-specific histology, particularly frequently observed yet relatively inconsequential lesions, is critical to health assessment. Histological tissue collection protocols should be established for use during the rare opportunities that arise to obtain fresh tissues from healthy mysticetes other than bowhead whales. Standardized histological collection protocols should be considered a routine and important part of health assessment efforts, have species-specific importance, and may have significant comparative value.

Concentrations of toxicants in the blood and tissues of whales may fluctuate with increased industrial development and deposition of contaminants in the Arctic. In our studies, many of the elemental analyzed, including Zn, Cd, Se, and tHg, accumulated with age. This emphasizes the effect of ontogeny on elemental concentrations. A significant association was found between the concentrations of renal and hepatic Cd and the amount of fibrosis in the lung and kidney. Both Cd concentration and fibrosis were found to be strongly associated with age. Age is therefore a confounding factor in this association. These results highlight the importance of age as a variable in both toxicology research and histological assessment. The accurate and precise determination of individual ages versus the use of length as a proxy for age in marine mammals will help to clarify some of these associations. Additionally, connecting element concentrations with quantifiable effects in tissues (via histology, biomarkers, etc.) may help elucidate the connection between toxicity and effects seen at the population level in these species.

Biomarkers can be a useful component of health assessment, as they often allow for indirect inference of health, contaminant status, etc., and commonly can be attained through blood or biopsy sampling of live animals. The establishment of baseline values for hematology, nutritional indicators, contaminant concentrations and tissue appearance are key to the interpretation of these values. The lack of controlled experimental trials in marine mammals may hinder the full understanding of the biomarker response to stressors, but this alone should not disqualify their utility. Most biomarker information in marine mammals has been gathered retrospectively from wild populations, and data from rodent models are supportive of their use. However, questions remain as to how specific the effects of stressors are for the biomarkers we have measured and with what degree of accuracy these biomarkers represent the stressors being measured. The use of biomarkers in conjunction with other studies that provide insight into the health and contaminant exposure of the individual is recommended. Results should be interpreted with caution, and biomarkers should be measured on a long-term basis. It is important for additional data to be gathered, as this will add to our knowledge of biomarker dynamics and the value of these biomarkers as indicators, not only of contaminant load, but of ongoing (e.g., offshore industrial activities) and emerging (e.g., climate change) potential stressors.

Nutritional indices may hold the most promise as indicators of health, especially if a more precise methodology for the measurement of the hypodermal layer of blubber is developed. The hypodermal layer appears to be the most metabolically active and is most often present in recently weaned juvenile whales. The disappearance or decline in the thickness of this layer over several seasons may make a useful commentary on the productivity of the region and the condition of the animals examined (especially calves and possibly their mothers).

Marine mammal blubber is influenced by the quality and quantity of the animal's diet; therefore, there is considerable value in using blubber thickness, lipid and blubber collagen percent (BCP) measurements in future health assessment projects. Data from the detailed sampling scheme employed here have shown that these measurements should be made at several sites on the whale. My results also indicate the need for additional investigations into the anatomy of blubber (gross and histological), the composition and dynamics of its framework (collagen, elastin) and the effects of different nutritional states on blubber

collagen. Improved methodology for the measurement of the hypodermal layer and investigations into the dynamics of this region would be especially helpful. Clear and agreed upon definitions of the term "blubber" are needed as well, especially when samples are being collected from large cetaceans that are likely to have a well-developed hypodermal layer. Defining these layers will help to clear up uncertainty that may exist with respect to sampling region. The use of appropriate histologic and morphologic characteristics will facilitate greater understanding of this important tissue in cetaceans.

In retrospect, the development of improved methods of hypodermal blubber measurement along with close attention to collection of samples from adult whales (especially males) are the two main things I would have changed. As mentioned above, I suspect the hypodermal layer to be an important indicator of nutritional status, particularly in juvenile whales, where this layer is well-developed. This age class is over represented in our sample. Intensive collection from older whales should be made a priority, as it was frustrating at times to be missing key tissues from otherwise complete tissue sets. Rigorous collection protocols and a priority status placed on older whales are needed to round out sample collection. Tissues from older animals also lend an important dimension to these studies, as aged animals exposed to their surroundings on a long-term basis are likely to be quite different from tissues sampled from juvenile whales.

The bowhead whale is a species of great cultural and subsistence value to Northern Alaskan native groups. The whales examined in this study were healthy, as defined by biomarkers and lack of pathological lesions; however, we recommend limiting consumption of renal tissue due to high Cd content. The liver is not commonly eaten in Barrow and Kaktovik, so this is not as much of an issue as it may be in other villages in northern Alaska and Russia, where this tissue may be consumed to some extent. Liver is high in Cd, but may also expose consumers to toxic levels of vitamin A even when eaten in small amounts. The return of health assessment information to the community is an important component of this work and will be accomplished through presentations made to the general public in subsistence villages and informational handouts detailing results and issues that are of importance to subsistence lifestyles.

In closing, I strongly recommend conducting an intensive health assessment effort every 4 years (as an adjunct to the bowhead whale census conducted by the North Slope Borough Department of Wildlife

Management) to facilitate the measurement of temporal changes that may occur in response to industrial development/anthropogenic contaminant input into the region and global climate change. This interval may need to be adjusted if changes are noted in the area and/or in the whales landed during the hunt. Large whales of advanced age should be given priority status for tissue collection and morphometrics. These whales should always be sampled in order to augment the data collected from these underrepresented age classes. Health assessment is complementary to ongoing population monitoring efforts and is essential to ensure that the species will be viable for generations to come and to assure subsistence users of the robust and healthy status of this stock of whales.

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